THE ANTIHEMOSTATIC PROFILE OF VITAMIN C: MECHANISMS THAT UNDERLIE THE TECHNICAL APPLICATION OF A PHYSIOLOGICAL MOLECULE

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__Abstract__: The potential of antioxidants as tools for decreasing the incidence of diseases, including cardiovascular events, is of growing interest. Some antioxidants (e.g. vitamin E and acetyl-salicylic acid) have been described as effective on cardiovascular diseases with mechanisms that differ from other scavenging agents. Currently, vitamin C is used to open occluded long-term central venous catheters, which avoids the process of reinserting a new one and injuring the patient. In this work, we investigated the vitamin C antihemostatic profile by evaluating its effects on the coagulation process. We used different assays, including prothrombin time (PT), activated partial thromboplastin time (aPTT), thrombin time (TT) and ancrod time tests. We also examined the overall pH disturbance caused by vitamin C at different concentrations and its effect on the thrombin-initiated fibrin polymerization assay. Our results revealed a significant anticoagulant activity of vitamin C at high plasma concentrations (surpassing the normal 100 µmol/L ratio) in a cell-independent mechanism. Our results suggest that vitamin C may affect blood coagulation by a direct impairment of fibrin assembly and further formation of a cohesive clot microstructure. This study supports the literature that points to the antihemostatic ability of antioxidant agents, and clarifies the mechanism of vitamin C in opening occluded long-term central venous catheters.

__Key words__: Vitamin C; ascorbic acid; antioxidant; blood; coagulation

INTRODUCTION

Vitamin C is a water-soluble antioxidant and an enzyme cofactor that can be found in many biological systems of different origin [1-3]. Most plants and animals synthesize ascorbic acid, with the exception of apes and humans that cannot synthesize this vitamin due to a lack of gulonolactone oxidase. Currently the human population obtains this molecule mainly through fruits, vegetables and pharmaceutical supplements [4]. International guidelines, such as the United States Department of Agriculture and National Cancer Institute publications, recommend the ingestion of an average of five fruits daily (200-300 mg of ingested vitamin C, depending on the fruit consumed) [5].

Current literature suggests that vitamin C reduces health disorders such as cardiovascular disease by trapping organic free radicals while deactivating excited oxygen species to prevent tissue damage [6]. Several reports show the importance of oxidative stress as a key factor in the development of cardiovascular diseases [7]. In addition, the literature points to the positive effects of polyphenols and other antioxidants on lipid profile, rates of myocardial infarction and cardiomyocyte apoptosis, also decreasing the overall risk of cardiovascular disease [8-10], prompting to their cardioprotective ability [11,12]. Recent studies show that vitamin C may develop a protective role in ischemic heart disease due to its ability to increase the overall

time of thrombus formation, decrease platelet aggregation rates and superoxide anion generation [13,14].

The influence of antioxidants in hemostasis has been known for decades. This knowledge extended the use of vitamin K-dependent carboxylase antagonists (vitamin E) to influence fibrinogen acetylation [15]. They affect fibrin clot structure and its susceptibility to lysis (e.g. a high-dose treatment with acetyl salicylic acid) [15-17].

Macedo and Kang reported the clinical usage for vitamin C in flow-recovery from occluded long-term central venous catheters, instead of streptokinase or tissue plasminogen activator, establishing its application as an ongoing practice in hospitals for over 13 years [18]. These results revealed the influence of high doses of vitamin C on the fibrinolytic process, however, no studies have clarified its role in blood coagulation and/or its direct influence in the plasma phase of clot formation. In this work, we analyzed the effects of vitamin C in several cell-independent *in vitro* assays, with the aim of examining the direct influence of this molecule at high-concentrations in the plasma phase of clot formation.

MATERIALS AND METHODS

In order to evaluate the vitamin C anticoagulant profile, we performed different assays, including prothrombin time (PT), activated partial thromboplastin time (aPTT), thrombin time (TT) and ancrod time tests. Different concentrations of vitamin C (0.033, 0.16, 0.3, 1.16 and 3.33 mg/mL) were pre-incubated (0.10 min and 30 min) in citrated plasma pools (n=6) from donors of the Antônio Pedro University Hospital (HUAP) in compliance to the protocol approved by the Human Research Ethics Committee of the Federal Fluminense University Medicine School (ID number: 177/11). All tests were performed using the coagulometer Coab Lab IV – Labor Lab®. We assessed the overall pH disturbance ratio for each sample of vitamin C at different concentrations during the assays using a pH meter [19]. Minimal disturbance was observed for all samples, which presented pH values ranging from 7 to 7.4. Further, we performed a thrombin-initiated fibrin polymerization assay to assess the role of vitamin C in fibrin network formation.

Activated partial thromboplastin time assay (APTT)

Activated partial thromboplastin time was measured as previously described by Martinichen-Herrero [20] to evaluate the clotting of human plasma initiated through the intrinsic coagulation pathway. The reactant (aPTT-EA, TECO GmgH®, Germany) and plasma samples were preheated at 37ºC and plasma was incubated with vitamin C at different concentrations (0.033-3.3 mg/mL) for 2 min. Then, the reactant was added to the preheated plasma and incubated for 2 min at 37ºC. Finally, 0.025 M calcium chloride (100 μL) was added to the sample to trigger the coagulation cascade, and the clot formation time was assessed.

Prothrombin time assay (PT)

Prothrombin time (PT) was used to evaluate human plasma clotting initiated through the extrinsic coagulation pathway. As for the APTT, the reactant for PT (TeClot, TECO GmgH®, Germany) and plasma samples were preheated at 37ºC and plasma was pre-incubated with vitamin C at different concentrations (0.033-3.3mg/mL) for 2 min. Further, PBS (100 μL) was added to the preheated plasma and incubated for 2 min at 37ºC. Finally, 100 μL of the reactant were added to the sample to trigger the coagulation cascade, and clot formation time was monitored [20].

Thrombin time assay (TT)

We performed TT assays by pre-incubating 200 μL of plasma at 37ºC for 2 min with 3 μL of vitamin C at different concentrations (0.033-3.3mg/mL) as previously described [20]. Then, 100 μL of bovine thrombin (Sigma Aldrich, code T4648) was added to the sample to trigger reaction and onset fibrin clot formation.

Thrombin-initiated fibrin polymerization (TIFP) assay

The TIFP assay was performed as described in the literature [21,22] to evaluate the formation of thrombin-catalyzed-fibrin clots. Briefly, 5 μL of bovine thrombin was incubated in 10 mM HEPES buffer with different concentrations of vitamin C in a final volume of 100 μL at pH 7.4. After 20 min at 37°C, the rate of fibrin polymerization was measured at 405 nm using a microplate reader (Thermoplate TP-Reader®) to evaluate fibrin network formation by global turbidity values. The polymerization reaction was triggered by the addition of 50 μL of bovine fibrinogen (4 mg/mL) (Sigma Aldrich, Code: T4648).

Ancrod time

Ancrod is a thrombin-like serine protease that differs from thrombin by releasing only fibrinopeptide A instead of both fibrinopeptide A and B from cleaving fibrinogen. It does not activate factor XIII, which results in an unstable clot due to a poor fibrin network. In order to evaluate the influence of vitamin C in the ancrod-catalyzed fibrin clot formation, 200 μL of plasma were preheated at 37°C for 2 min containing 3 μL of vitamin C at the same concentrations as used in the thrombin time assays (0.033-3.3mg/mL). Then, 100 μL of preheated ancrod (0.33 mg/mL) were added to the sample to trigger reaction and onset of fibrin clot formation [23].

Statistical analysis

All statistical analyses are expressed by representative means±SD of three independent experiments analyzed by ANOVA, followed by Tukey's multiple comparison test, using SPSS 14.0® for Windows. P<0.05 was considered statistically significant.

RESULTS

Activated partial thromboplastin time (APTT)

According to the experimental data, vitamin C has no significant influence on the intrinsic pathway of the coagulation cascade, even at high concentration levels or high incubation periods (Fig. 1). Therefore, a potential vitamin C inhibitory coagulation mechanism does not affect plasmatic proteins from the blood coagulation cascade's intrinsic pathway. These data suggest that the direct effect of vitamin C on thrombus formation must occur either uniquely *in vivo* or affect the coagulation cascade from the extrinsic or common pathways as suggested by Wannamethee et al. [14].

Prothrombin time (PT)

The prothrombin time (PT) data showed a slight increase in prothrombin time when increasing the vitamin C concentration in plasma. This effect has no relation to the pre-incubation time (Fig. 2). These data pointed to the influence of vitamin C in clot formation from a very specific mechanism that significantly af-
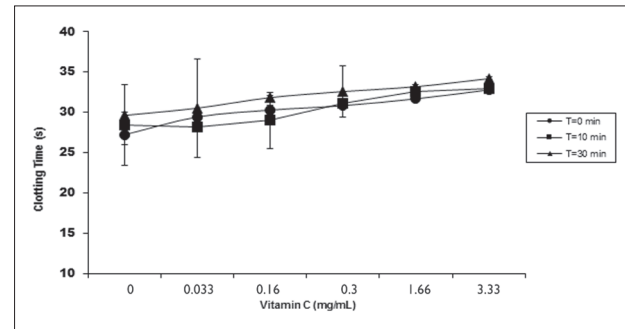

Fig. 1. Comparison of the activated partial thromboplastin time from human plasma at different concentrations of vitamin C (0.033-3.33 mg/mL) and pre-incubation times (T = 0.10 and 30 min).
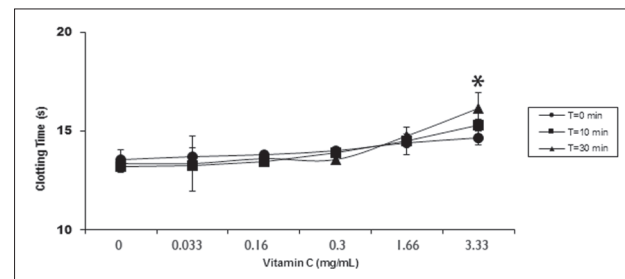

Fig. 2. Comparison of the prothrombin time from human plasma at different concentrations of vitamin C (0.033-3.33 mg/mL) and pre-incubation times (T = 0.10 and 30 min). An asterisk above individual vitamin C concentrations indicates statistical significance (p<0.05) relative to a vitamin C concentration of 0 mg/mL.

fects the extrinsic pathway on the coagulation cascade at concentrations above 1.66 mg/mL, when triggered by thromboplastin.

Thrombin time (TT)

Thrombin time assay revealed a dose-dependent activity of vitamin C in the common pathway of the coagulation cascade, with statistical significance observed at 1.66 and 3.33 mg/mL final concentrations (Fig. 3). Different concentrations of vitamin C influenced the thrombin time assay. Our results also revealed that the incubation time affected the inhibitory activity of vitamin C, pointing to a kinetic-like behavior.

Thrombin-initiated fibrin polymerization

In order to evaluate the direct anticoagulant activity of vitamin C and its influence on fibrin network formation, we performed thrombin-initiated fibrin polymerization assays. Incubation with 3.33 mg/mL of vitamin C decreased the global fibrin polymerization promoted by thrombin in an ascorbate-free sample at 37ºC (Fig. 3). These data corroborate the data of Zbikowska et al. [24] in which ascorbate reduced the fibrin polymerization from gamma-irradiated fibrinogen, while reducing the incorporation of carbonyl groups to the fibrinogen molecule. This leads to an effective protection of fibrinogen from gamma irradiation-induced oxidation. However, our results suggest that the fibrinogen oxidation generated by thrombin may be impaired by the natural antioxidant profile of ascorbate.

Ancrod time (AT)

To analyze the antioxidant influence of vitamin C in clot formation, we measured the clot formation time catalyzed by ancrod as an alternative clot-promoting agent to thrombin. Our results showed that vitamin C still affects the overall clot formation even when using ancrod, a thrombin-like enzyme from a venomous snake. This result implies that vitamin C does not have a molecular specificity for thrombin but rather directly affects the normal fibrin assembly. These re-
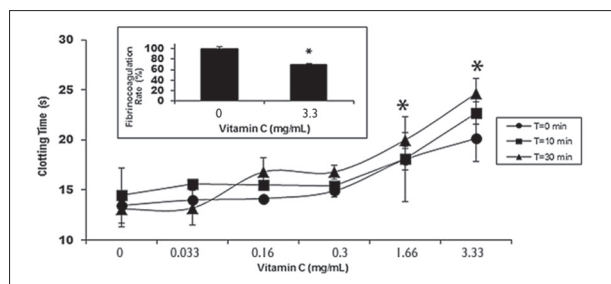

Fig. 3. Comparison of the thrombin time from human plasma at different concentrations of vitamin C (0.033-3.33 mg/mL) and pre-incubation times (T = 0.10 and 30 min). Inset box: comparison of fibrin clot formation shown in the complete absence of and at high ascorbate concentration (3.33 mg/mL), revealing a direct impairment of fibrin network). An asterisk above individual vitamin C concentrations indicates statistical significance ($p<0.05$) relative to a vitamin C concentration of 0 mg/mL.

Fig. 4. Comparison of ancrod time from human plasma at different concentrations of vitamin C (0.033-3.33 mg/mL) and pre-incubation times (T= 0.10 and 30 min). An asterisk above individual vitamin C concentrations indicates statistical significance ($p<0.05$) relative to a vitamin C concentration of 0 mg/mL.
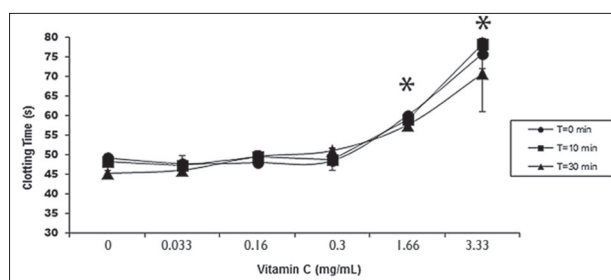
sults are similar to thrombin time data, with statistical significance observed for ancrod clotting time also at the highest concentrations of vitamin C tested (1.66 mg/mL and 3.33 mg/mL) (Fig. 4). This supported the potential of vitamin C to impair fibrin clot formation despite of its cleavage origin.

DISCUSSION

In this work, we evaluated the anticoagulant profile of vitamin C using different coagulation assays. Our results revealed that vitamin C directly affects clot formation, probably through a molecular mechanism that impairs the assembly of a cohesive fibrin network. According to our data, the mechanism is independent

of the clot-promoting agent as vitamin C affected both thrombin- and ancrod-catalyzed assays. These data suggest that vitamin C promotes the impairment over clot formation, possibly due to the poor assembling of fibrin protofibrils and further fiber network formation.

Overall, our data is similar to that of Therani et al. [17], who studied the influence of high antioxidant-agent doses in normal fibrin clot formation. Ascorbate is the physiological form of vitamin C at pH 7.4 and displays an important chemical role as a donor antioxidant agent [25,26]. Our results suggest that ascorbate may affect fibrin network assemblage and final microstructure through a direct impairment of protofibrils and fiber formation. These results are reinforced by previous data from Macedo and Kang [18] that described a reduction in protofibrils and fiber formation on clots originating from blood treated with vitamin C.

The literature indicated that coagulation clots stay under intense lysis conditions [27]. Thus, the effect of vitamin C on fibrin network microstructure observed herein led to a fragile final product that was susceptible to lyses, similar to that described by Therani et al. [17] who studied acetylsalicylic acid. Our data support the positive effect of vitamin C in cardiovascular diseases such as thrombosis described previously [12].

Wannamethee et al. [14] reported that a fragile fibrin network would improve lysis after the activation of fibrinolytic proteins, such as tissue plasminogen activator/plasmin, where thrombus formation time would be considerably reduced along with superoxide generation. In such a context, according to the literature, platelet aggregation could be affected by ascorbate through the interaction of fibrinogen with the integrin glycoprotein complex present in platelet glycoprotein IIB/IIIA [28].

In this work, we observed a distinct influence of vitamin C on clot formation in a dose-dependent manner that relies on other parameters, such as incubation time (but without affecting the pH even at high concentrations). According to our data, the anticoagulant activity of vitamin C does not rely on the establishment of an acidic medium that could lead to acidosis and further promote endogenous fibrinolytic
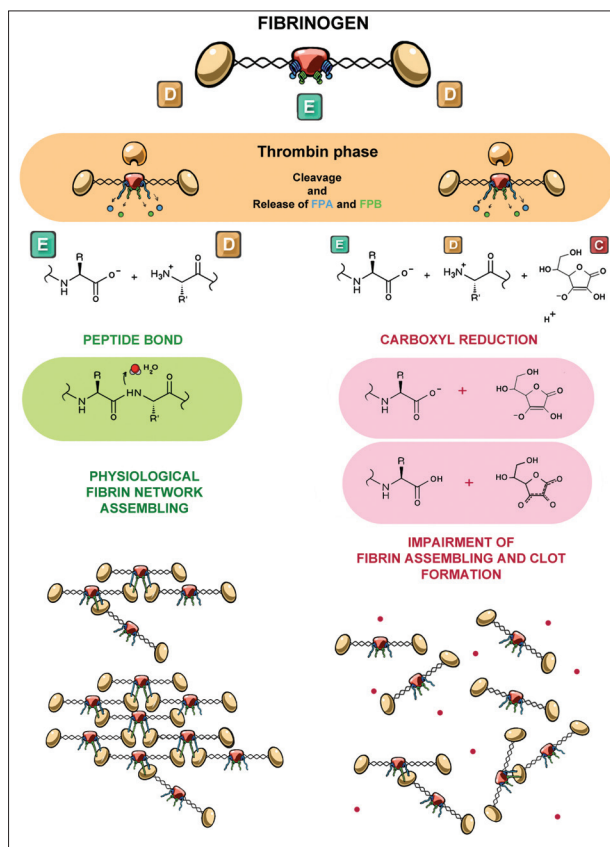

Fig. 5. Proposed molecular mechanism of vitamin C impairment of fibrin clot formation at pH 7.4.

events *in vivo*, as suggested previouly [29]. We propose an anticoagulant mechanism based on the antioxidant potential of the active agent, where the substrate cleavage of the E-portion of fibrinogen by thrombin removes the N-terminal 16 and 14 peptides known as fibrinopeptides A (FPA) and B (FPB), respectively (Fig. 5). This removal would lead to the release of carboxyl groups in the E nodule (E) in fibrin, which are necessary for the interaction with the D nodule (D) of adjacent fibrin monomers. Furthermore, the release of FPA would promote protofibril formation, while the release of FPB would promote lateral aggregation of preexisting protofibrils, fiber formation and a cohesive fibrin network. Therefore, ascorbate (C), the soluble form of vitamin C in plasma, would donate a hydrogen atom at pH 7.4 to the oxidizing radical of (E) to produce the resonance-stabilized tricarbonyl ascorbate free-radical while thermodynamically im-

pairing the interaction between E and D terminal residues of fibrin monomers and their further linkage by factor XIII transglutaminase [30]. This might prevent the formation of covalent bonds that are important in promoting a final cohesive fibrin network structure, leading to an unstable clot that is more susceptible to physiological lysis (Fig. 5).

CONCLUSIONS

Our results suggest a direct influence of vitamin C in the final pathway of clot formation. Its anticoagulant profile probably relies on its antioxidant potential, which impairs the physiological assembly of fibrin monomers and thus, the formation of a cohesive network of fibrin fibers. Unlike the mechanisms observed for other antioxidants, such as vitamin E (through vitamin K antagonism) and acetylsalicylic acid (through fibrinogen acetylation), vitamin C might prevent fibrin assembly by direct impairment of the interaction between the E and D terminal residues of fibrin monomers at pH 7.4, while forming the resonance-stabilized tricarbonyl ascorbate free-radical from its stable ascorbate form. This work lends support to the proposed antihemostatic ability of this antioxidant, and to its potential use against thrombotic situations and thrombogenic conditions.

Authors' contributions: Plínio Cunha Sathler wrote the protocol, and the first draft of the manuscript. André Luiz Lourenço. Ana Paula Gentile Arêas and Max Seidy Saito performed the statistical and the experimental analysis. Carlos Rangel Rodrigues, Lúcio Mendes Cabral, Helena Carla Castro managed the analyses of the study and the literature searches. Hye Chung Kang designed the study. All authors read and approved the final manuscript.

Conflict of interest disclosure: No potential conflicts of interest relevant to this article were reported.

Ethical approval: This study was submitted to the Research Ethics Committee of the Faculty of Medicine of the University Hospital Antônio Pedro / HUAP Fluminense Federal University, and approved according to Resolution 196/96 of the Ministry of Health (CMM/HUAP, REGISTRATION ID 177/11).

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