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Review

Ligand binding to fibrinogen influences its structure and function

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Summary. Fibrinogen is a plasma protein that is highly susceptible to oxidation. Because of this chemical modification, fibrinogen acquires thrombogenic characteristics under different pathophysiological conditions. Increased carbonyl content and reduced porosity impair the degradation of formed fibrin mediated by plasmin. Fibrinogen is capable of interacting with many proteins, ions, and small molecules. These interactions can modify the functions of this protein. The discovery of new binding partners that may protect fibrinogen from harmful oxidation and, thus, preserve its normal function is essential. Some of the newly detected interactions between fibrinogen and small, natural bioactive molecules, together with the influence of these interactions on the structure and function of fibrinogen, will be presented in this text.

Keywords: bilirubin, dihydrolipoic acid, fibrinogen, protein function, protein-ligand interaction, protein structure, resveratrol.

FIBRINOGEN

Fibrinogen or coagulation Factor I is the main protein involved in coagulation. In healthy individuals, its concentration ranges from 2 to 4 g/L (Bialkower et al. 2019). Fibrinogen is a large fibrillar glycoprotein with a molecular mass of 340 kDa and an (AαBβγ)2 fold (Mosesson 2005). As shown in the crystal structure of fibrinogen (Fig. 1), the central structural motif is a coiled-coil formed by the involvement of all chains. At the N-terminus, six chains of fibrinogen form the so-called central E-domain, while two terminal D-domains at both ends of fibrinogen are created by Bβ- and γ-chains. Two Aα-chains extend from D-domains, and on their ends, they form αC-domains. These αC-domains are in close proximity to the central E-domain. Fibrinopeptides A and B (FpA and FpB, respectively) are cleaved by thrombin, inducing the formation of insoluble fibrin (Fig. 1) (Weisel and Litvinov 2013).

There are four sites on fibrinogen that are N-glycosylated, one on each β- (Asn364) and γ-chain (Asn52). Dominant glycans on fibrinogen are complex, bi-antennary types that end with one or two sialic acids (Adamczyk et al. 2013). Fibrinogen is mainly synthesised in hepatocytes. Biosynthesis starts with the individual expression of each chain, followed by the assembly of the whole protein in the endoplasmic reticulum. Half-molecular structures, AαBβγ, are formed first, followed by formation of the entire molecule. After glycosylation, fibrinogen enters circulation (Kattula et al. 2017).

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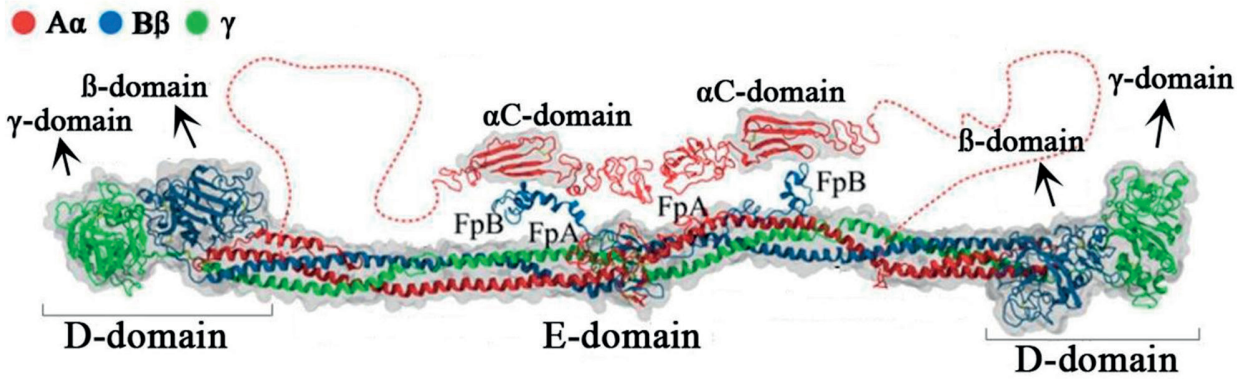

Fig. 1. Structure of fibrinogen. Individual fibrinogen chains and domains are marked (modified from Weisel and Litvinov 2013).

FUNCTION OF FIBRINOGEN

Fibrinogen is involved in both primary and secondary haemostasis (Mosesson 2005). Coagulation and thrombolysis are two highly regulated and connected processes (Mosnier and Bouma 2006). Different factors affect the balance between these two processes, including: coagulation factors, blood platelets, and endothelial cells. The entire coagulation process can be divided into two types, known as primary and secondary haemostasis.

Primary haemostasis (Fig. 2) involves formation of platelet clots at the site of blood vessel injury. When exposed to collagen, fibronectin, laminin and von Willebrand factor, platelets become activated. This activation leads to changes in their shape and activation of several proteins (Ia/IIa and IIb/IIIa integrins) at the platelet surface. Fibrinogen can bind to two IIb/IIIa integrins, thus strengthening the interactions between activated platelets (Broos et al. 2011).

Secondary haemostasis (Fig. 3) involves the formation of fibrin that assembles into a mesh that mechanically sup-

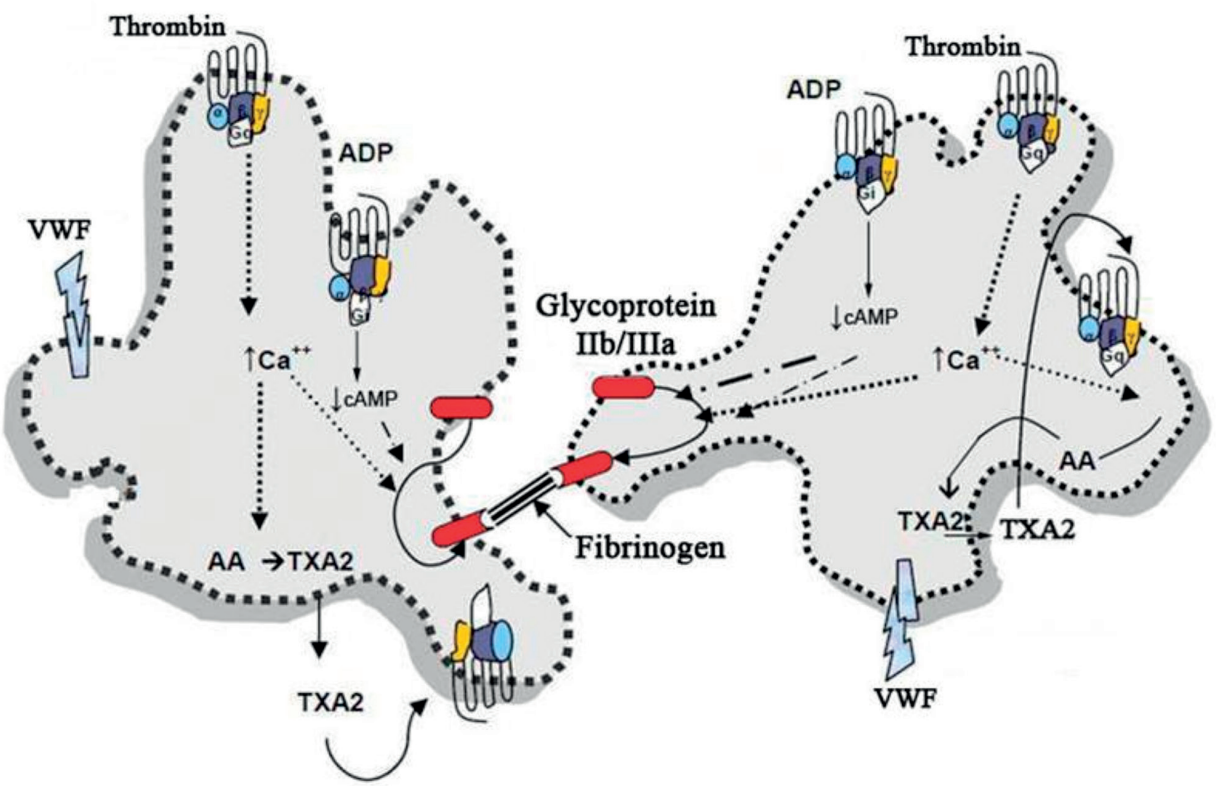

Fig. 2. The process of primary haemostasis.

ports the primary platelet clot. The process of fibrin formation can be initiated by two (albeit interconnected) different pathways, and involves activation of several coagulation factors that together lead to the formation of a thrombin activation complex that is capable of activating thrombin from its pro-form. Thrombin supports fibrin formation from fibrinogen and activates factor XIII, which becomes factor XIIIa. Factor XIIIa is involved in the covalent linkage of fibrin monomers in growing fibres (Palta et al. 2014).

OXIDATION OF FIBRINOGEN

Fibrinogen is a plasma protein that is highly susceptible to oxidation (Shacter et al. 1994). It has been proposed that fibrinogen may scavenge oxidative species and protect other proteins from oxidation (Undas and Ariëns 2011). Hypochlorous acid (HOCl), a metabolic product of myeloperoxidase, was shown to specifically oxidase Met residues in fibrinogen, leading to the formation of dense fibrin with thin fibres and a reduction of the rate of lysis by plasmin (Weigandt et al. 2012). Peroxynitrite can also chemically modify Tyr residues on fibrinogen, which make it more thrombogenic (Nowak et al. 2007). Oxidised fibrinogen has thrombotic characteristics, i.e. the resulting fibrin that is formed cannot be efficiently degraded. During oxidative stress related pathologies, fibrin clots formed from oxidised fibrinogen display reduced porosity (Martinez et al. 2013). Alterations in the thickness of fibrin fibres also occurs (Undas and Ariëns 2011): some pathologies are characterised by thinner fibres (diabetes mellitus, peripheral arterial disease, myocardial infarction), whereas others are characterised by thicker fibres (acute myocardial infarction, acute stroke, venous thromboembolism, end-stage renal disease). Under pathological conditions such as cirrhosis, formed fibrin clots are less porous, although morphologically they appear the same as those of healthy individuals (Hugenholtz et al. 2016). Reduced porosity is considered to be a consequence of increased carbonylation of fibrinogen.

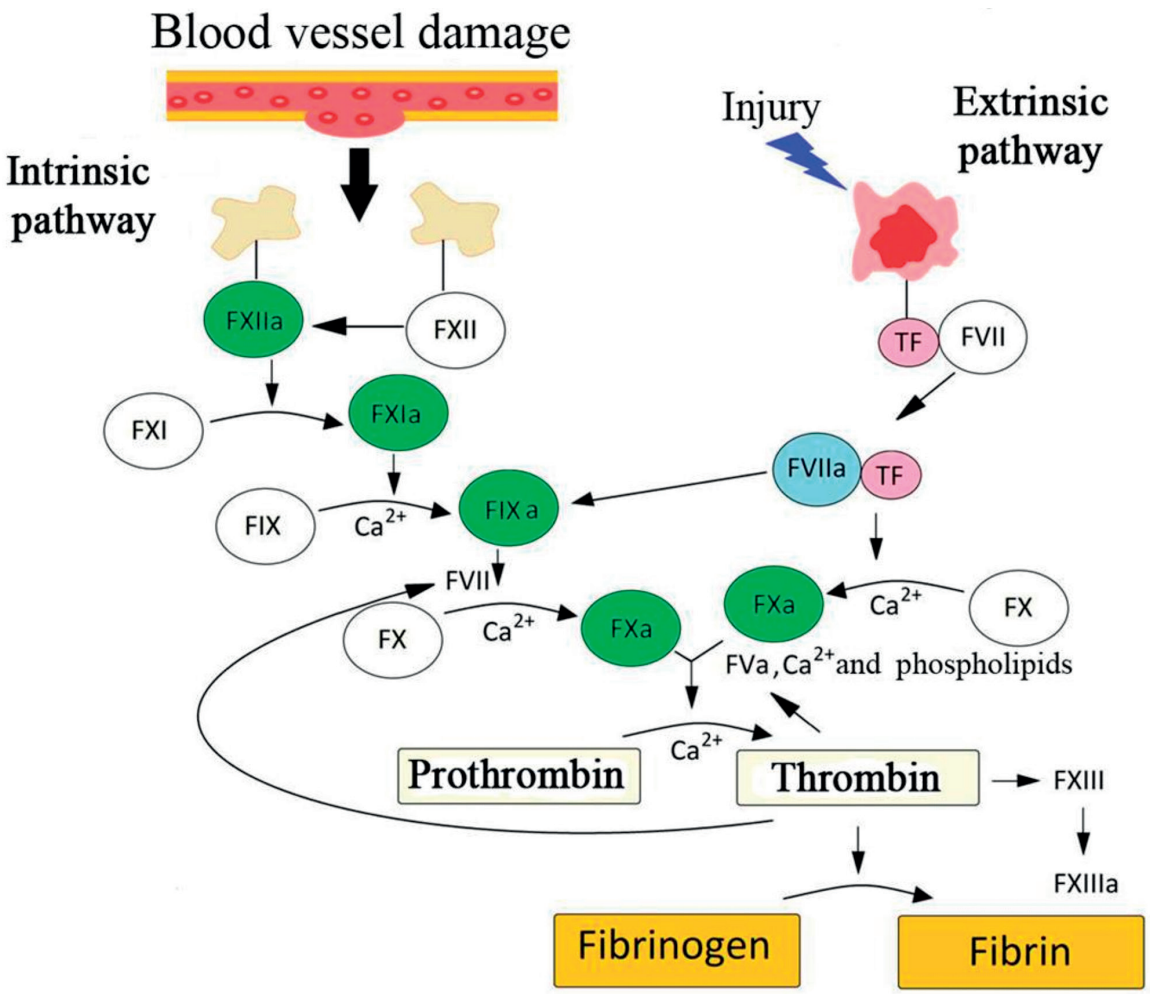

Fig. 3. Schematic presentation of secondary haemostasis with fibrin formation.

Carbonylation increases the hydrophobicity of fibrin and prevents efficient penetration and degradation by plasmin (Lisman and Ariëns 2016).

BINDING OF BILIRUBIN, RESVERATROL AND DIHYDROLIPOIC ACID TO FIBRINOGEN

The binding of different small molecules to fibrinogen has been characterised by spectrofluorimetry. Fibrinogen contains many Trp and Tyr residues (Kollman et al. 2009) and thus has a robust intrinsic fluorescence signal. Because of this, minimal concentrations of fibrinogen are needed for fluorimetric experiments. Increasing concentrations of bilirubin, resveratrol and dihydrolipoic acid (DHLA) quench the intrinsic fluorescence of fibrinogen in a dose-dependent manner (Fig. 4A), where results obtained from addition of resveratrol are presented. The linearity of Stern-Volmer plots obtained from fluorescence spectra intensities indicate that only one type of quenching was present (Fig. 4B) (Lakowicz 2016). Further analysis showed that the quenching rate constant for all tested molecules was higher than the diffusion rate of the biomolecules, which on average is approximately 10^{10} $M^{-1}s^{-1}$. This data strongly suggests that fluorescence quenching in fibrinogen occurs upon complex formation with the tested molecules (Minic et al. 2018). A double logarithmic plot was used to calculate affinity constants (Fig. 4C), and all three tested molecules bind to fibrinogen with moderate affinities (Gligorijević et al. 2019, 2020a, 2020b).

None of the tested ligands had any significant effect on the structure of fibrinogen. This finding is not surprising as fibrinogen is a large, fibrillar protein, and binding of small molecule(s) may not be sufficient to induce significant conformational changes. Figs 5A and B show representative results obtained from CD spectrometry analysis of the structure of fibrinogen in the absence and presence of increasing concentrations resveratrol: the near-UV region was examined for tertiary structure analysis and the far-UV region was used for secondary structure analysis. Both near- and far-UV CD spectra were almost unaltered by resveratrol binding. Similar results were obtained for bilirubin binding to fibrinogen (Gligorijević et al. 2019, 2020a).

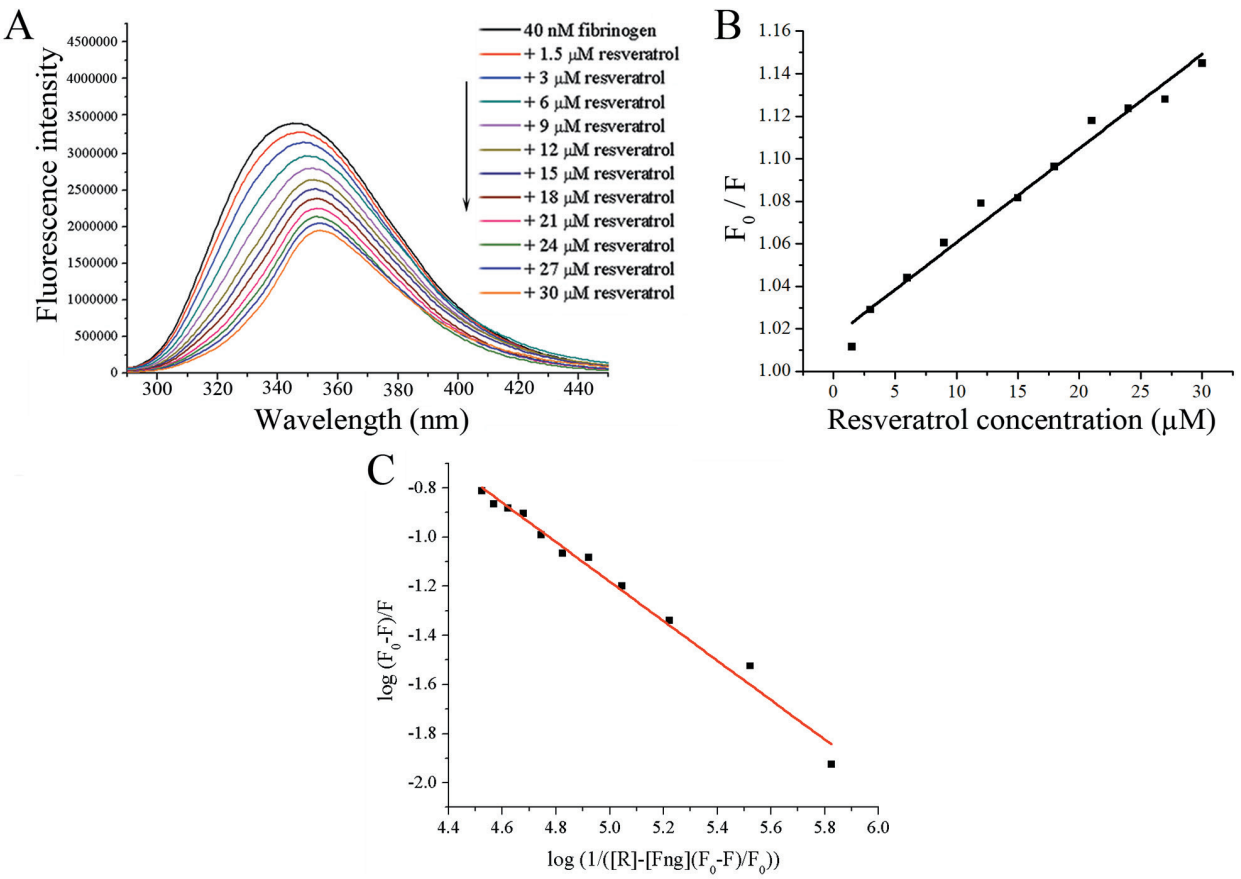

Fig. 4. Quenching of fluorescence signal originating from fibrinogen in the presence of increasing concentrations of resveratrol (**A**); Stern-Volmer plot used for the calculation of Stern-Volmer constant and determination of quenching type (**B**); Stern-Volmer plot used for the calculation of binding constants between fibrinogen and resveratrol (**C**).

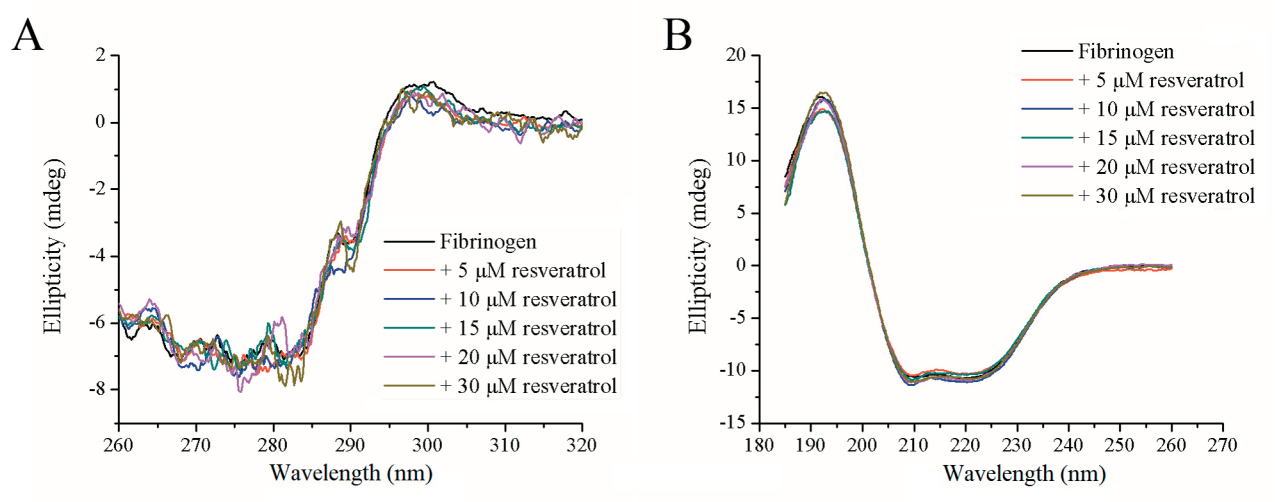

Fig. 5. CD spectra of fibrinogen in the presence and absence of resveratrol recorded in the near- **(A)** and far-UV region **(B)**.

The secondary structure of fibrinogen in the presence of DHLA was analysed using FTIR (Fig. 6). Deconvolution of the obtained Amide I band signal originating from C=O stretching vibrations in protein amide bonds was used to calculate the percentage of secondary structure motifs. Similarly to the previous findings, no significant changes in secondary structures were observed, implying that the structure of fibrinogen remains almost unaltered upon binding of DHLA (Gligorijević et al. 2020b).

Comparison of the absorption spectra of bilirubin and DHLA alone and in the presence of fibrinogen revealed changes in both spectral shape and absorption maxima. In the case of bilirubin (Fig. 7A), the absorption spectra became wider and exhibited a redshift, while in DHLA, a blue shift was observed (Fig. 7B). In the presence of fibrinogen, the fluorescence emission intensity of resveratrol increased upon fibrinogen binding, followed by a significant blue shift of the peak (Fig. 7C). These spectral results confirm the formation of complexes between fibrinogen and the tested molecules, indicating that binding alters both conformation and the polarity around them.

2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH) is a widely used oxygen free radical inducer in redox-related experiments. In its presence, the intrinsic fluorescence of fibrinogen decreases over time as a consequence of changes to the protein structure due to oxidation. This reduction of intrinsic fluorescence is hindered in the presence of resveratrol and DHLA, indicating that these molecules can protect fibrinogen from harmful oxidation, as can be seen in Figures 8A and 8B. Upon bilirubin binding, reduced carbonylation of fibrinogen was observed (Gligorijević et al. 2019). Thus, the binding of all three ligands to fibrinogen expresses

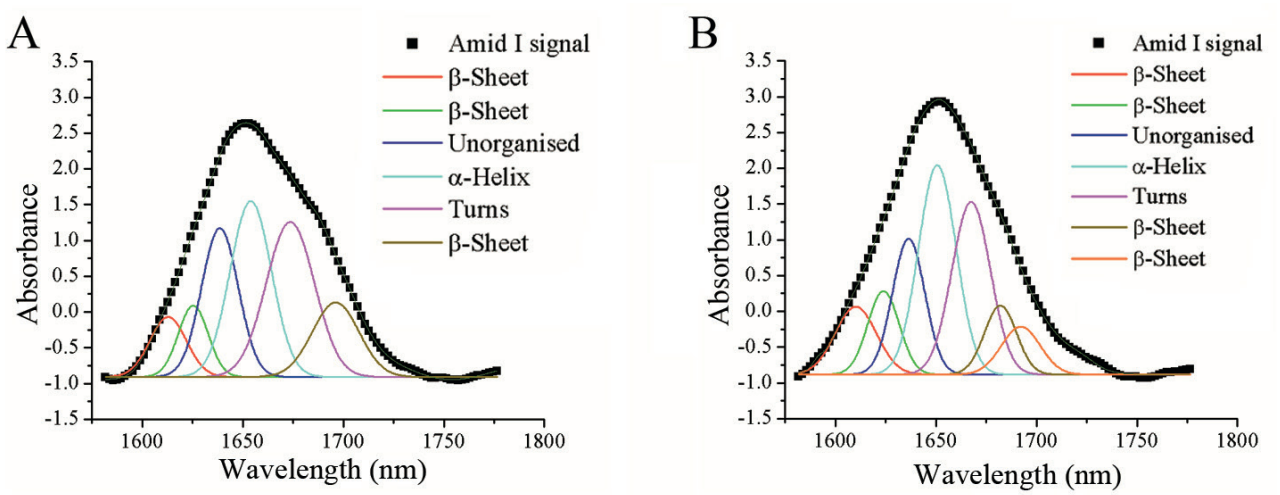

Fig. 6. Secondary structure analysis of fibrinogen using FTIR in the absence of DHLA **(A)** and 1:10 molar ratio of fibrinogen and DHLA **(B)**.

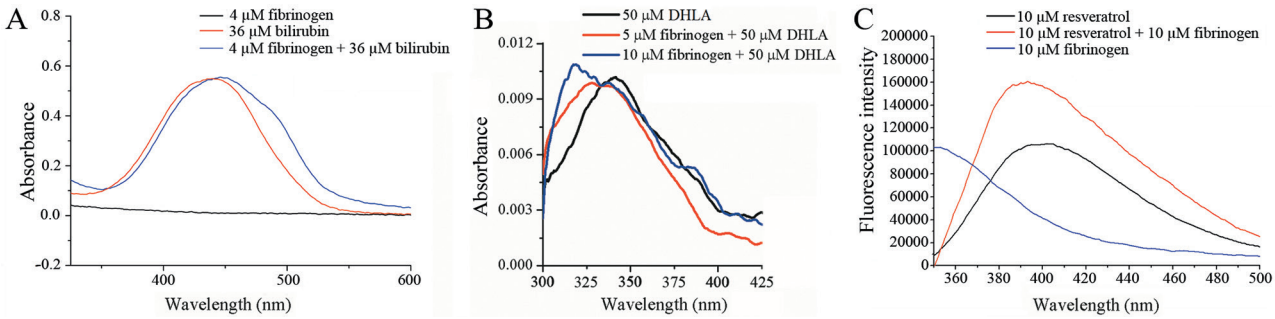

Fig. 7. Absorption spectra of bilirubin (A) and DHLA (B) in the presence and absence of fibrinogen. Emission spectra of resveratrol (C) in the presence and absence of fibrinogen.

an antioxidative protective effect on the protein. Bilirubin and DHLA also influence the formation of fibrin, as shown by a turbidimetric assay. In this test, the formation of fibrin fibres is accompanied by light scattering, which is monitored as increased absorbance at a specific wavelength. Thicker fibres scatter more light than thinner fibres. Thus, the change in absorbance during fibrin formation correlates with fibre thickness (Tilley et al. 2011). Our results show that in the presence of bilirubin (Fig. 8C) and DHLA (Fig. 8D), thicker fibrin fibres are formed (Gligorijević et al. 2019, 2020b).

While it is already known that resveratrol has no direct influence on fibrin formation (Malinowska and Olas 2010), mutual protection from oxidation was detected between resveratrol and fibrinogen: the stilbene compound protects fibrinogen from oxidation, and the same protective effects were provided by fibrinogen for resveratrol (Fig. 9A). The poor bioavailability of resveratrol under physiological conditions is one of the main reasons that many of the positive effects of resveratrol observed *in vitro* are not always reproducible *in vivo* (Gligorijević et al. 2021). Because of

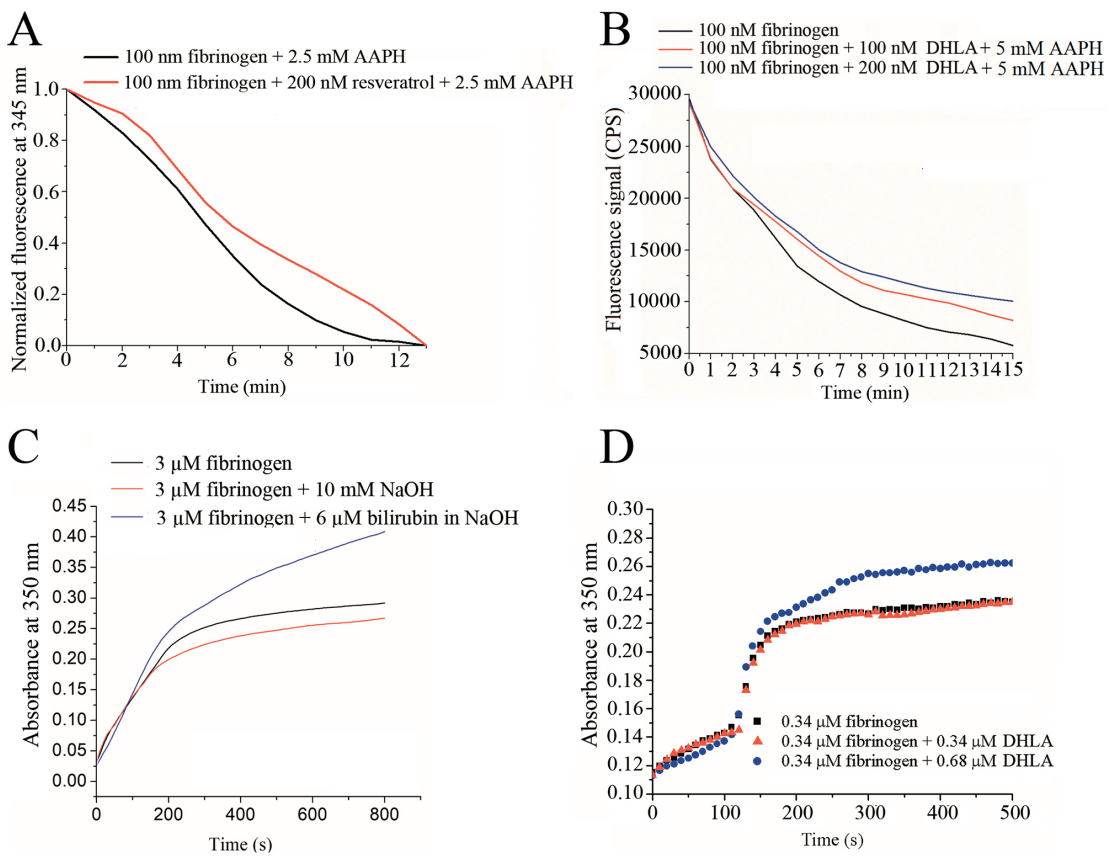

Fig. 8. Protection of fibrinogen from oxidation induced by AAPH by resveratrol (A) and DHLA (B). Influence of bilirubin (C) and DHLA (D) on the thickness of fibrin fibres.

this, intensive research efforts are underway to try to find ways to increase resveratrol bioavailability. According to our results, the solubility of resveratrol in an aqueous environment and, thus, its potential bioavailability, is increased in the presence of fibrinogen (Gligorijević et al. 2020a). These data were obtained by dispersing large amounts of resveratrol in a buffer with and without fibrinogen and recording the UV absorption spectra of the resulting mixtures. The intensity of the absorption maximum of resveratrol was higher in the presence of fibrinogen, indicating a higher concentration of solubilised resveratrol (Fig. 9B). Similar results were obtained for β-lactoglobulin (Liang et al. 2008). The reducing power of resveratrol in the absence and presence of fibrinogen was also investigated by measuring potassium hexacyanoferrate (III) reduction. The antioxidative potential of resveratrol remains unchanged in the presence of fibrinogen (Fig. 9C). This finding is important considering that the biological activity of resveratrol relies mainly on its antioxidative potential.

CONCLUSIONS

The obtained results show that fibrinogen protects resveratrol from oxidation, increases its solubility and does not interfere with its antioxidative potential. At the same time, resveratrol protects fibrinogen from oxidation without affecting fibrin formation. It can be hypothesised that resveratrol may prevent or alleviate thrombotic-related complications, in which oxidised fibrinogen has an important role. The resveratrol/fibrinogen interaction outcome may offer one possible explanation for the mechanism responsible for the famous French paradox that relies on red wine consumption and the polyphenols associated with it.

Bilirubin and DHLA have antioxidative effects on fibrinogen that are similar to resveratrol. However, in contrast to resveratrol, these compounds modulate fibrin formation, suggesting a potential physiological relevance of these interactions, as thicker fibrin fibres form in their presence. Possible supplementation of these two molecules may be

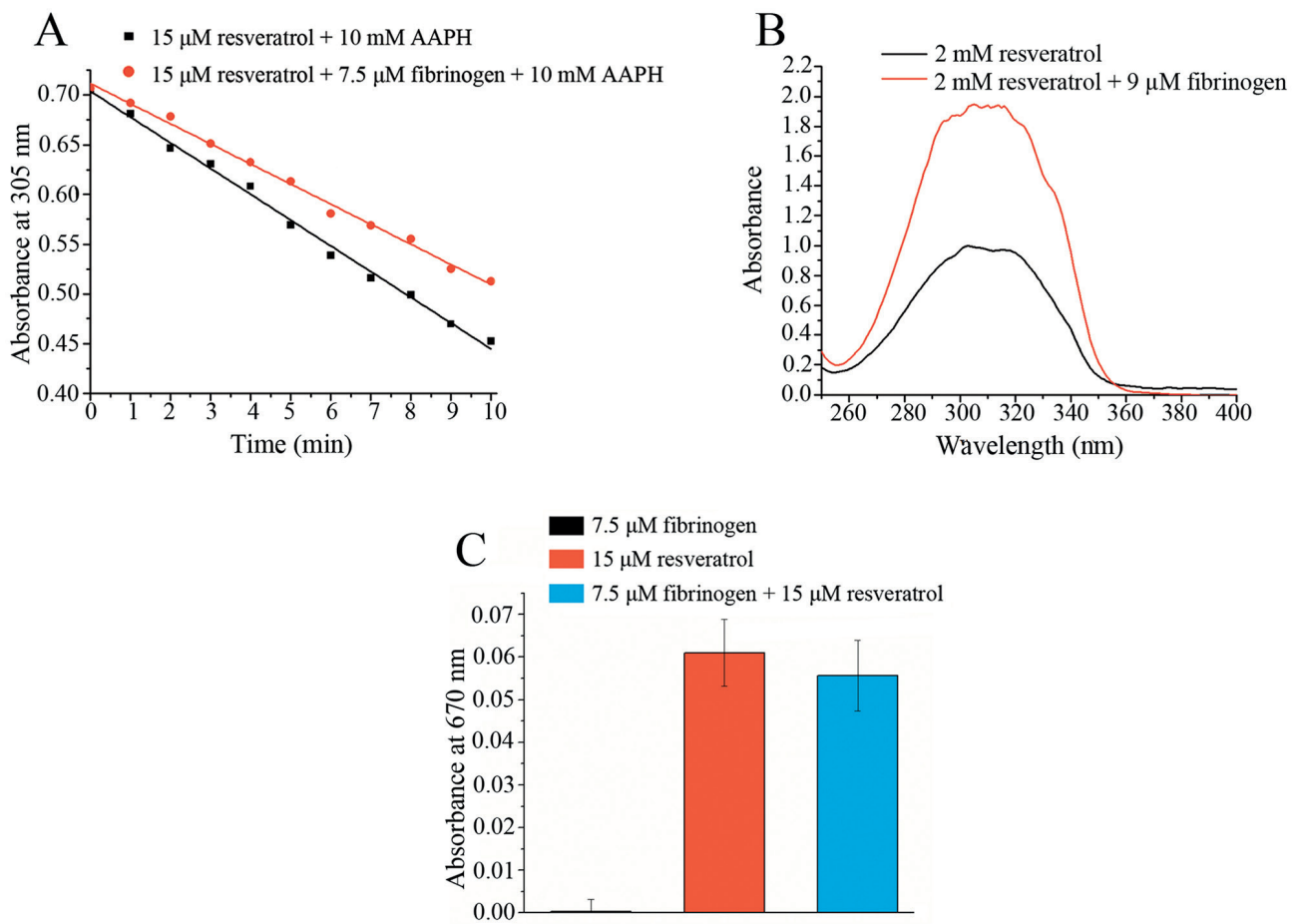

Fig. 9. Protection of resveratrol from AAPH-induced oxidation by fibrinogen **(A)**, the solubility of resveratrol in the presence and absence of fibrinogen **(B)** and reducing the potential of resveratrol in the presence and absence of fibrinogen **(C)**.

considered for persons with an increased risk of developing thrombotic complications, particularly those whose fibrin is characterised by increased oxidative modification and the formation of thinner and less porous fibres.

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REFERENCES

Adamczyk B, Struwe WB, Ercan A, Nigrovic PA, Rudd PM. 2013. Characterisation of fibrinogen glycosylation and its importance for serum/plasma N-glycome analysis. Journal of Proteome Research. 12(1):444–454.

Bialkower M, McLiesh H, Manderson CA, Tabor RF, Garnier G. 2019. Rapid paper diagnostic for plasma fibrinogen concentration. Analyst. 144(16):4848–4857.

Broos K, Feys HB, De Meyer SF, Vanhoorelbeke K, Deckmyn H. 2011. Platelets at work in primary hemostasis. Blood Reviews. 25(4):155–167.

Gligorijević N, Minić S, Robajac D, Nikolić M, Ćirković Veličković T, Nedić O. 2019. Characterisation and the effects of bilirubin binding to human fibrinogen. International Journal of Biological Macromolecules. 128:74–79.

Gligorijević N, Radomirović M, Rajković A, Nedić O, Ćirković Veličković T. 2020a. Fibrinogen increases resveratrol solubility and prevents it from oxidation. Foods. 2020(9):780.

Gligorijević N, Šukalović V, Penezić A, Nedić O. 2020b. Characterisation of the binding of dihydro-alpha-lipoic acid to fibrinogen and the effects on fibrinogen oxidation and fibrin formation. International Journal of Biological Macromolecules. 147:319–325.

Gligorijević N, Stanić-Vučinić D, Radomirović M, Stojadinović M, Khulal U, Nedić O, Ćirković Veličković T. 2021. Role of Resveratrol in prevention and control of cardiovascular disorders and cardiovascular complications related to COVID-19 disease: mode of action and approaches explored to increase its bioavailability. Molecules. 2021(26):2843.

Hugenholtz GCG, Macrae F, Adelmeijer J, Dulfer S, Porte RJ, Lisman T, Ariëns RAS. 2016. Procoagulant changes in fibrin clot structure in patients with cirrhosis are associated with oxidative modifications of fibrinogen. Journal of Thrombosis and Haemostasis. 14(5):1054–1066.

Kattula S, Byrnes JR, Wolberg AS. 2017. Fibrinogen and fibrin in hemostasis and thrombosis. Atherosclerosis, Thrombosis and Vascular Biology. 37(3):e13–e21.

Kollman JM, Pandi L, Sawaya MR, Riley M, Doolittle RF. 2009. Crystal structure of human fibrinogen. Biochemistry. 48(18):3877–3886.

Lakowicz JR. 2006. Principles of Fluorescence Spectroscopy. 3rd ed. New York: Springer US.

Liang L, Tajmir-Riahi HA, Subirade M. 2008. Interaction of beta-lactoglobulin with resveratrol and its biological implications. Biomacromolecules. 9(1):50–56.

Lisman T, Ariëns RAS. 2016. Alterations in fibrin structure in patients with liver diseases. Seminars in Thrombosis and Hemostasis. 42(4):389–396.

Malinowska J, Olas B. 2010. Effect of resveratrol on hemostatic properties of human fibrinogen and plasma during model of hyperhomocysteinemia. Thrombosis Research. 126(5):e379–e382.

Martinez M, Weisel JW, Ischiropoulos H. 2013. Functional impact of oxidative posttranslational modifications on fibrinogen and fibrin clots. Free Radical Biology and Medicine. 65:411–418.

Minic S, Stanic-Vucinic D, Radomirovic M, Radibratovic M, Milcic M, Nikolic M, Cirkovic Velickovic T. 2018. Characterisation and effects of binding of food-derived bioactive phycocyanobilin to bovine serum albumin. Food Chemistry. 239:1090–1099.

Mosesson MW. 2005. Fibrinogen and fibrin structure and functions. Journal of Thrombosis and Haemostasis. 3(8):1894–1904.

Mosnier LO, Bouma BN. 2006. Regulation of fibrinolysis by thrombin activatable fibrinolysis inhibitor, an unstable carboxypeptidase B that unites the pathways of coagulation and fibrinolysis. Atherosclerosis, Thrombosis and Vascular Biology. 26(11):2445–2453.

Nowak P, Wachowicz B. 2002. Peroxynitrite-mediated modification of fibrinogen affects platelet aggregation and adhesion. 13(5-6):293–299.

Palta S, Saroa R, Palta A. 2014. Overview of the coagulation system. Indian Journal of Anaesthesia. 58(5):515–523.

Shacter E, Williams JA, Lim M, Levine RL. 1994. Differential susceptibility of plasma proteins to oxidative modification: Examination by western blot immunoassay. Free Radical Biology and Medicine. 17(5):429–437.

Tilley D, Levit I, JA Samis JA. 2011. Development of a microplate coagulation assay for factor V in human plasma. Thrombosis Journal 9(1):11.

Undas A, Ariëns RAS. 2011. Fibrin clot structure and function: a role in the pathophysiology of arterial and venous thromboembolic diseases. Atherosclerosis, Thrombosis and Vascular Biology. 31(12):e88–e99.

Weigandt KM, White N, Chung D, Ellingson E, Wang Y, Fu X, Pozzo DC. 2012. Fibrin clot structure and mechanics associated with specific oxidation of methionine residues in fibrinogen. Biophysical Journal. 103(11):2399–2407.

Weisel JW, Litvinov RI. 2013. Mechanisms of fibrin polymerisation and clinical implications. Blood. 121(10):1712–1719.