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Effect of Oxidized Fibrinogen on Hemostatic System: In Vitro Study

Sermin Tetik, PhD,[1] Kurtulus Kaya, MSc,[1] and Turay Yardimci, PhD[1]

Abstract

Standard coagulation assays were performed with control and oxidized fibrinogen (Fg), using prothrombin time (PT; 12.5 ± 0.4 vs 25 ± 0.8 seconds, $P < .001$) and activated partial thromboplastin time (aPTT; 33 ± 2.5 vs 63 ± 4.7 seconds, $P < .001$). Fibrin clot (MA), clot formation initiation (r), and rate of clot lysis (LY30) were measured, a reflection exposure of Fg to Fe^{3+}/ascorbate oxidative system by thrombelastograph (TEG) analysis (0, 6, 12, 24, and 48 hours, 6.2 ± 1.3 vs 5.5 ± 1.2, 4.3 ± 1.0 [$P < .01$], 3.9 ± 1.6, 3.2 ± 0.8, [$P < .001$]). Maximum amplitude level was found to be lower than control (69.1 ± 7.2 vs 67.9 ± 12.4, 64.0 ± 11.4, 60.2 ± 21.2, 42.2 ± 15.2, $P < .001$). The lysis rate was changed according to oxidation time between Fg exposed to Fe^{3+}/ascorbate and control exposed to Fe^{3+}/ascorbate for the same treatment time (1.9 ± 0.71 vs 7 ± 0.5, 1.6 ± 0.1, 1.2 ± 0.5, 0.9 ± 1.3, $P < .001$). We revealed dysregulation of hemostatic system with contribution of oxidized Fg, which was in direct proportion to the intensity of Fg oxidation.

Keywords

oxidized fibrinogen, thrombelastography, hemostasis

Introduction

Epidemiological studies have been linked to the development of pathological states, and several diseases (eg, tumors, diabetes mellitus, and inflammation) are accompanied by oxidative stress process.[1] When free radicals oxidize important molecules such as fibrinogen (Fg), albumin of the blood or cell, those components lose the ability to function normally. Numerous studies indicate that increased production of free radicals causes or accelerates the nerve cell injury and leads to disease.[2-4] Plasma proteins are exposed to oxidants in a variety of circumstances in vivo, such as during tissue injury and inflammation.[5]

Fibrinogen is an adhesive plasma protein that plays a pivotal role in hemostasis.[6] In response to blood vessel injury, platelets initiate clotting by simply adhering to and spreading on Fg and/or extracellular matrix components readily exposed at the site of vascular damage.[7,8] The platelet adhesion and aggregation require binding of Fg and/or other large adhesive proteins by the platelet integrin receptor glycoprotein (Gp) IIb/IIIa.[9,10]

Recent studies showed that the lysine residues of Fg are the functional groups most susceptible to oxidative modification on Fg.[11] On exposure to a radical generating system, Fg may undergo various posttranslational modifications that results in functional alteration of the Fg.[2] Oxidized Fg may occur in many pathological conditions such as atherosclerosis, hemorrhagic injury, and sepsis.[12] The first biological outcome of oxidative changes to Fg structure is the inhibition of thrombin-catalyzed clot formation.[13] Furthermore, Fe^{3+}/ascorbate–oxidized Fg showed a distinct capacity from the native molecule to bind platelet receptor Gp IIb/IIIa.[14,15]

Modification of Fg with oxidative stress may occur in many pathological conditions. Upper systemic levels of native and oxidized Fg may mediate the risk for disease conditions; therefore, both Fg modification and its functional results that might contribute to disease pathogenesis have been discussed. The relationship between Fg dysfunction and oxidized Fg remains unclear.

In this study, we studied the in vitro effects of oxidized Fg on fibrin clot strength and coagulation cascade.

Materials and Methods

Preparation of Oxidized Fg

Fibrinogen (Sigma, St Louis, Missouri) was oxidized in vitro by Fe^{3+}/ascorbate, according to the procedure described by Levine.[16] Commercial Fg was dissolved at a concentration of

[1] Department of Biochemistry, Faculty of Pharmacy, Marmara University, Istanbul, Turkey

Corresponding Author:
Sermin Tetik, Department of Biochemistry, Faculty of Pharmacy, Marmara University, Tibbiye Cad No 49, 34668 Haydarpasa, Istanbul, Turkey.
Email: stetik@marmara.edu.tr

2.8 mg/mL in 25 mmol/L *N*-(2-hydroxethyl) piperazine-*N*′-(2,2-ethanesulfonic acid) (HEPES), 25 mmol/L ascorbate, and 20 mmol/L $MgCl_2$ (pH 7.2). Treatment of Fg by 100 μmol/L Fe^{3+}/ascorbate oxidative system was performed at different incubation times (0, 6, 12, 24, and 48 hours) at 37°C. At the desired times, a sample was withdrawn and mixed with the iron chelator EDTA (Sigma, St Louis, Missouri; final concentration: 1 mmol/L). The 0th time was evaluated as a control Fg. The excess of reagent was removed by dialysis at room temperature in 10 mmol/L phosphate buffer (2.5 mmol/L phosphate-buffered saline [PBS], pH 7.4). Oxidized and control Fg samples were filtered through a 0.045-mm membrane, aliquoted, and stored at room temperature.[17] In spectrophotometric analysis, differences between control and oxidized Fg on exposure to the Fe^{3+}/asorbate oxidative system were detected. Fibrinogen was exposed to 0, 6, 12, 24, and 48 hours incubation times, with Fe^{3+}/ascorbate (100 μmol/L per 75 mmol/L) in vitro. Dityrosine formation on oxidized Fg was evaluated by spectrophotometry (characteristic emission at max λcm 380 nm).[17]

Blood Collection

After obtaining the institutional ethics committee approval, written informed consent was obtained from all the participants participating in this study. Blood was obtained from 10 healthy volunteer donors, who had not received any medications in the 2 weeks before blood collection. The same volunteers (19-26 years old) were included for the study of both control and oxidized Fg. Nine parts of blood from a venepuncture were mixed with 1 part of 0.1 mol/L citrate (Merck, Darmstadt, Germany.) as anticoagulant in plastic tubes.

Standard coagulation assays (MTI, MT4-C model, 4-channel semiotomatical instrument, Turkey) were performed with fresh plasma, using prothrombin time (PT) and activated partial thromboplastin time (aPTT; rabbit origin thromboplastin and the other reagents from Trinity Biotech, Wicklow, Ireland., by mechanical methods).

Thrombelastograph Measurement

Thrombelastograph measured the kinetics of clot formation and growth as well as the strength and stability of the formed clot. The kinetics of clot formation stands for the adequacy of quantitative factors, and the strength and stability of the formed clot stand for the ability of the clot to do the work of hemostatic system. Thrombelastograph (5000 TEG Thrombelastograph Analyzer, Haemoscop Corporation, IL) was performed after quality control procedures as described[18] and the following TEG parameters were recorded: r represents the time to initiation of clot formation, the maximum amplitude represents the maximum clot strength, and lysis rate-30 represents the rate of cloth lysis 30 minutes after MA. All measurements were performed when nasopharyngeal temperature was >35°C.

In separate pilot experiments, we empirically evaluated the TEG using varying incubation times (0, 6, 12, 24, and 48 hours), oxidized Fg, and different predilutions on human blood to optimize the sensitivity of the method. In this study, we evaluated 3 different final concentrations of control and oxidized Fg (0.5, 1, and 1.5 mg/mL), according to the study by Belisario et al.[13] We derieved the final concentration of oxidized and control Fg as 1.5 mg/mL on TEG analysis. However, the best and replicated result was decided 0.5 mg/mL at the final concentration of oxidized and control Fg only in coagulation assay. For the measurements, citrated whole blood was immediately diluted with control and oxidized Fg (final concentration 1.5 mg/mL)[13] to a total volume of 300 μL and incubated for 10 minutes at 37°C. Coagulation was initiated by addition of 20 μL of 0.2 mol/L $CaCl_2$ solution and the results were recorded. All samples were measured as duplicates on the same TEG instrument.

On the 2-channel TEG equipment, we investigated the oxidized Fg as well as the nonoxidized Fg. The activated experiments were performed to stimulate in vitro conditions in the blood in which Fg triggers coagulation. The thrombelastographic investigations were carried out immediately after collection of the blood samples.

Whole Blood Coagulation Analyses

Measure of aPTT. Blood samples collected in vacuum-tubes with citrate to arrest coagulation by binding calcium. To activate the intrinsic pathway, phospholipid, an activator, and calcium were mixed into the plasma, and oxidized and control Fg (final concentration 0.5 mg/ml) were added and slightly mixed. The time was measured until clot forms.

Measure of PT. Protrombin time measurement was used to determine the clotting tendency of blood. Blood was drawn into the test tube containing citrate that acts as an anticoagulant by binding the calcium in a sample. The blood was mixed, then centrifuged for 20 minutes at 2000*g* to separate blood cells from plasma. The plasma was analyzed by an automated instrument at 37°C. Tissue factor was added; the prothrombin time is the time it takes for the plasma to clot after addition of tissue factor. The measurement of prothrombin time was repeated after the addition of oxidized and control Fg into plasma. Prothrombin time was recorded.

Control and oxidized Fg were included in the plasma for PT and aPTT tests or whole blood for TEG analysis and the results were immediately recorded. The platelet count was adjusted to 2.5×10^8 platelet/mL.

Statistical Analysis

The results for the 10 samples were expressed as mean value ± standard deviation. All samples and standards were run in duplicate. Multivariate analyses were used on TEG data to evaluate the influence of platelet number and Fg level on the TEG parameters. Comparison between the 2 groups was tested

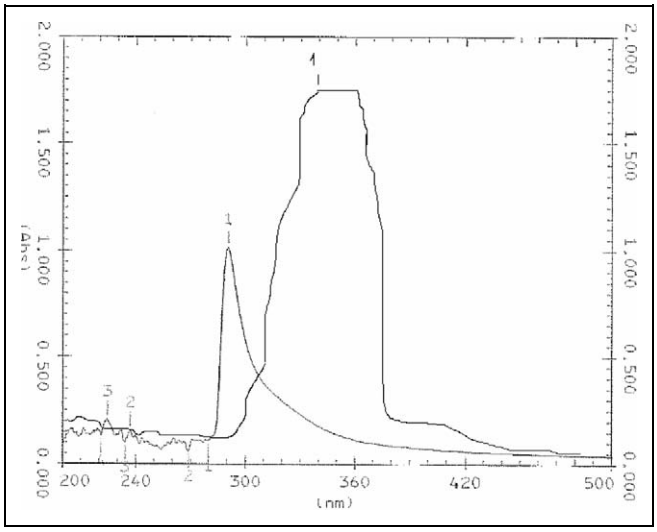

Figure 1. Spectrophotometric assay of dityrosine formation on control and oxidized fibrinogen.

by Student *t* test. *P* values lower than .05 were considered significant.

Results

The characteristic emission of Fg exposed to the Fe+3/ascorbate oxidative system (48 hours) at λcm 325 nm is a marker of the presence of dityrosine in oxidized Fg (Figure 1). This indicates that the exposure of Fg to the Fe^{+3}/ascorbate system induces conformational changes, which result in increased emission spectra of aromatic amino acids on Fg.

Prolonged thrombin time and defective polymerization of fibrin monomers due to the time of Fg exposure to the oxidative system was seen at standart coagulation assay. The values were represented in Table 1. The levels of PT and aPTT increased as a function of Fg oxidative damage.

The characteristics of clot formation and growth as well as the strength and the stability of the formed clot were measured using the Thrombelastograph Hemostasis System. We performed the quality control procedures as described by Ak et al.[18]

Measured TEG parameters were significantly different between effect of oxidized and control Fg. Hemostatic characteristics in blood with oxidized Fg were changed in an antithrombotic way (Table 2). Oxidized Fg (48 hours) had a minimal initiation rate of clot formation than control ($P < .001$). The initiation phase of coagulation was correlated with "r" value. Moderated blood with oxidized Fg had reduced MA and lysis-rate 30 (LYS-30) levels compared with the control Fg. In the experiments, we evaluated the following changes in hemostatic level: an increase in clotting time, a decrease in the maximum amplitude, and a shortened rate of lysis upon exposure to the Fe^{3+}/asorbate oxidative system.

Table 1. Coagulation Test Results of Control (0th Hour) and Fe^{3+}/Ascorbate–Treated Fibrinogen (48 Hours)[a]

	Control Fibrinogen (0th Hour)	Oxidized fibrinogen (48 Hours)
PT (seconds)	12.5 ± 0.4	25.0 ± 0.8[b]
aPTT (seconds)	33 ± 2.5	63 ± 4.7[b]

NOTES: aPTT = activated partial prothrombin time; PT = prothrombin time.
[a] Treatment of fibrinogen by Fe^{3+}/ascorbate oxidative system was performed at different incubation times. Control and oxidized fibrinogen were added into the plasma (0.5 mg/mL final concentration). Each group consists of 10 healthy volunteer donors. Values are represented as mean ± SEM.
[b] $P < .001$, compared to control fibrinogen.

Discussion

This study was designed to examine the in vitro effect of oxidized Fg on hemostatic system and coagulation parameters. Dityrosine formation on Fg molecule was an important marker of Fe^{3+}/ascorbate–induced oxidative stress. In vitro and in vivo studies of protein modification by oxidant agents have demonstrated the formation of dityrosine cross-links.[19,20] Oxidative modification might contribute to the loss of tyrosine residue that converts to dityrosine.[21] We evaluated that dityrosine formation in oxidized Fg was significantly greater than that in control Fg, with further increase after incubation time. This conclusion is supported by an in vitro study, where metal ion catalyzed the oxidation of Fg-generated dityrosine, which results in diminished platelet aggregation and adhesion.[13]

Activated partial thromboplastin time and PT were used as screening tools to determine whether one has a coagulation problem. Activated partial thromboplastin time is a performance indicator, measuring the efficacy of both the "intrinsic" and the common coagulation pathways. It was used in conjunction with PT that measures the extrinsic pathway.

Based on our data, we suggest that oxidation of Fg may partially contribute to the impaired or decelerated clotting activity, due to incubation time. Our result is contradictory to the data showing that oxidation of Fg in vitro, by iron or by peroxynitrite, caused inhibition of clotting activity.[22,23] In addition, we showed that Fe^{3+}/ascorbate oxidative system repressed the binding capacity of Fg to own receptor on platelets.[15,24]

Covalent modification markedly impairs the function of Fg, when such modification affects Fg. Fibrinogen modifications that have been best studied are oxidation, nitration, homocysteinylation, and glycation. It appears that the clottability of Fg is maintained unless there is an intense degree of Fg modification; modest degrees of Fg modification can alter the rate of assembly of fibrin monomers into a fibrin clot and fiber structure.[25] Glycolaldehyde induces Fg posttranslational modification of lysine and arginine residues. This modification leads to the generation of persistent clots.[26]

However, citrullinated Fg markedly impairs the function of thrombin-catalyzed fibrin polymerization and also inhibits fibrin formation.[27]

Michelis et al[28] showed that in hemodialysis patients, administration of a high iron dose caused an increase in

Table 2. TEG Results of the Kinetics of Clot Formation, Clot Strength, and Clot Stability[a]

	Control Fibrinogen (0th Hour)	Oxidized Fibrinogen (6 Hours)	Oxidized Fibrinogen (12 Hours)	Oxidized Fibrinogen (24 Hours)	Oxidized Fibrinogen (48 Hours)
r (minutes)	6.2 ± 1.3	5.5 ± 1.2	4.3 ± 1.0[b]	3.9 ± 1.6[c]	3.2 ± 0.8[c]
MA (mm)	69.2 ± 7.2	67.9 ± 12.3	64.0 ± 11.4	60.2 ± 21.1	42.2 ± 21.1[c]
LY30 (%)	1.9 ± 0.7	1.7 ± 0.5	1.6 ± 0.1	1.2 ± 0.4	0.9 ± 1.3[c]

NOTES: LY30 = clot lysis; MA: maximum amplitude; r = the time initiation of clot formation; TEG = thrombelastograph.
[a] Treatment of fibrinogen by Fe^{3+}/ascorbate oxidative system was performed at different incubation times. Control and oxidized fibrinogen were added into the whole blood (1.5 mg/mL final concentration). Each group consists of 10 healthy volunteer donors. Values are represented as mean ± SEM.
[b] $P < .01$, [c] $P < .001$, compared to control group.

carbonyls per Fg. Based on these data, it appears that the conditions for severe in vivo protein oxidation are already present when iron complexes are administered.

In this study, posttranslational modification was created by metal catalyzed oxidative system on Fg molecule, and results were evaluated using conventional coagulation assay. When the oxidatively modified Fg was added in vitro to whole blood, PT and aPTT levels became prolonged. This indicates that the exposure of Fg to the Fe^{3+}/ascorbate system after 48 hours induces more prolonged thrombin time and defective aPTT levels than controls. Prolonged clotting time was not associated with "r" value on TEG analysis. Fibrinogen oxidation by incubation for more than 24 hours resulted in the formation of a decelerated clot and defective amino acid sequences on Fg structure, but TEG analysis and conventional coagulation assay demonstrated different results for the same clotting time.

Previous studies have identified that the oxidation of Fg by hypochlorite (HOCl) induced a decrease in the rate of fibrin polymerization, producing fibrin clots with decreased permeation properties.[22] In contrast, exposure of Fg to nitration conditions such as MPO/H2O2/NO2-, resulted in fibrin clots, thus increased level of Fg nitration may lead to a prothrombic state.[12] These findings are consistent with the previous studies showing that posttranslational modification of arginine, lysine, or tyrosine residues of Fg results in significant decreases in platelet adhesion and adenosine diphosphate (ADP)-stimulated aggregation.[13,25-28]

The effect of UV-irradiated Fg on blood coagulation revealed that disturbance in the formation of fibrin clot with oxidized Fg causes suppression of platelet aggregation mediated by collagen receptors and inhibition of aggregation associated with von Willebrand factor activity.[29]

Fibrinogen exposed to Fe^{3+}/ascorbate was added in vitro to blood and assessed using r, k, and MA parameters on TEG. Linear relationship was evaluated between levels of in vitro oxidation time of Fg and ratio of control Fg. r, k, and MA parameters were changed due to incubation time in Fg with oxidative system. When compared with the controls, samples displayed dysregulation of the initiation phase, a prolonged in the clot formation time, and a reduction in the maximum amplitude. These data showed that Fg is a target for modification by oxidative stress, with the potential dysregulation of these process in the coagulation pathway. Only the results of "r" value were contradictory with the previous studies and our coagulation assay of PTT.[1] However, our results may suggest a contradictory interaction between TEG and coagulation parameters as recorded in the literature.[30]

Conclusion

As a result, we evaluated the effect of oxidized Fg on hemostatic system in whole blood and showed that oxidized Fg produces alterations in blood coagulation parameters and this effect depends on the intensity of oxidation. However, direct evidence is still lacking that these modifications contribute to the decreased athero- and/or anti-atherothrombotic risk associated with pathological conditions. After all, the results of "r" value need to be developed.

Declaration of Conflicting Interests

The authors declared no conflicts of interest with respect to the authorship and/or publication of this article.

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