MECHANISM OF BLOOD COAGULATION BY NON-THERMAL ATMOSPHERIC PRESSURE DIELECTRIC BARRIER DISCHARGE

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Abstract

Mechanisms of blood coagulation by direct contact of non-thermal atmospheric pressure dielectric barrier discharge plasma are investigated. This study shows that no significant changes occur in pH or Ca2+ concentration of blood during discharge treatment. Thermal effects and electric field effects are also shown to be negligible. Investigating the hypothesis that the discharge treatment acts directly on blood protein factors involved in coagulation, we demonstrate aggregation of fibrinogen, an important coagulation factor, with no effect on albumin. We conclude that direct dielectric barrier discharge treatment triggers selective natural mechanisms of blood coagulation.

I. INTRODUCTION

Over the past few years non-thermal atmospheric pressure plasma has emerged as a novel promising tool in medicine. Compared to the effects of the more conventional thermal plasma [1], non-thermal plasma is selective in its treatment since it does not burn tissue. This enables many new medical applications including sterilization of living tissue without damage [2], blood coagulation [2], induction of apoptosis in malignant tissues and [3], [4] modulation of cell attachment [5], [6], [7]

Although blood coagulation by direct non-thermal plasma treatment has been reported before [2], the bio-chemical pathways (mechanisms) through which such coagulation occurs remain largely unclear. In this paper several possible mechanisms are investigated. We demonstrate that direct plasma triggers natural, rather than thermally induced, coagulation processes. We also demonstrate that release of Ca^{2+} and changes of blood pH level are insignificant. Instead, the evidence points to selective action of direct non-thermal plasma treatment on blood proteins involved in the natural coagulation processes.

The principle of operation of the discharge used in this work is similar to the Dielectric Barrier Discharges (DBD) introduced by Siemens [9] in the middle of 19th century. DBD occurs at atmospheric pressure in air or other gases when sufficiently high voltage of sinusoidal waveform or pulses of short duration are applied between two electrodes, when at least one of them is insulated [10]. The presence of an insulator between the electrodes prevents the build-up of high current. As a result, the discharge creates e-plasma (we use this term to avoid confusion with blood plasma) without substantial heating of the gas. This approach allows for treatment of biological samples without thermal damage while biological processes are initiated and/or catalyzed with the help of electrical charges [8].

II. MATERIALS, METHODS AND EXPERIMENTAL SET UP

In this paper we investigate mechanisms of blood coagulation by non-thermal atmospheric pressure plasma using an experimental setup similar to one previously described by the authors [2] and schematically illustrated in Figure 1. E-plasma is generated by applying alternating polarity pulsed voltage of ~35 kV magnitude (peak to peak) at 1 kHz frequency between the insulated high voltage electrode and the sample undergoing treatment. 1 mm thick polished clear fused quartz is used as an insulating dielectric barrier. The discharge gap between the bottom of the 1 mm thick quartz glass covering the copper electrode and top surface of the sample being treated was set to 2 mm. The diameter of the copper electrode employed was 2.5 cm. The power density of the discharge has been measured to be around 1.5 Watts/cm^2 using both electrical characterization and special calorimetric set-up [11].

All the treatments are at room temperature and atmospheric pressure and were carried out according to the same protocol. The control samples were placed in the same sample holder as the treated sample. The control sample was exposed to air for the same time as the treated samples were exposed to plasma. For plasma treatment of 500 µl of anti-coagulated whole blood a special sample holder was constructed. 25.4 mm tall polycarbonate plate was used as base and stainless steel rods were inserted into a 25.4 mm through-hole. 21.46 mm tall stainless steel rods were used.

For this study, de-identified whole blood samples with three different types of anticoagulants were obtained from Drexel University College of Medicine (DUCOM) Chemistry lab. 500 µl of the three different types of anti-coagulated whole blood, viz; heparinized whole blood; citrated whole blood and Ethylene Diamine Tetraacetic Acid (EDTA) whole blood were used. Each blood sample was treated for 5 s, 15 s, 30 s and 60 s to study the effect of e-plasma treatment. Changes in pH and Ca^{2+} concentration were determined immediately after treatment. pH of the sample was measured using a pH meter (Lazar Research Labs 6230n pH/mV/Temp meter) and pH micro-electrode

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(Lazar Labs PHR146XS). Ca^{2+} concentration was measured after the treatment using the above meter with a micro-ion selective electrode (Lazar Research Labs LIS-146CACM) and micro reference electrode (Lazar Research Labs LIS 146DJM). The mV values obtained were converted to molar values using software (Arrow Lab Systems™).

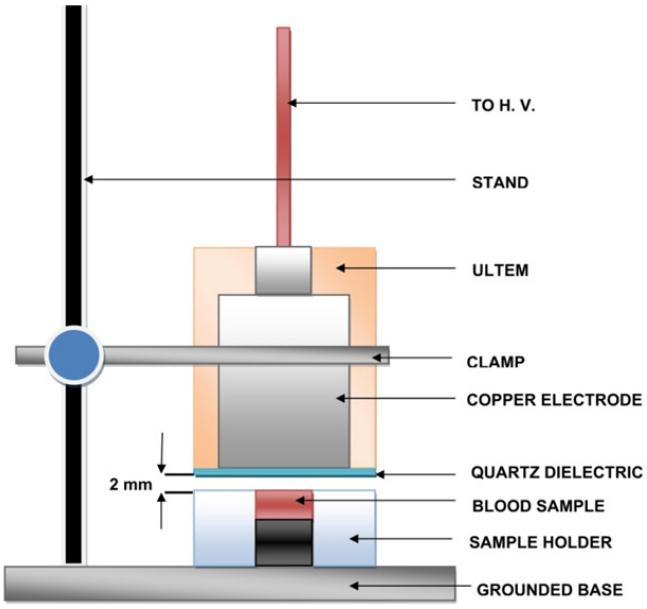


Figure 1. Schematic of the experimental setup showing the high voltage electrode and the sample holder

To study the effect of the non-thermal plasma exposure on albumin and fibrinogen, constituent proteins of blood plasma, purified lyophilized human serum albumin (Sigma-Aldrich, St. Louis, MO) was dissolved in trishydroxymethylaminomethane (TRIS) buffered saline to obtain albumin solution (physiological concentration of 2 g/l) at physiological pH 7.4. To prepare fibrinogen solution purified lyophilized fibrinogen from human plasma (Sigma-Aldrich, St. Louis, MO) was layered on top of warm (37ºC) 20 mM TRIS buffered saline (Sigma-Aldrich, St. Louis, MO) and slowly agitated for two hours to obtain fibrinogen solution (physiological concentration of 30 g/l) at physiological pH of 7.4.

Morphological evaluation of the clot layer, formed on the surface of blood post e-plasma treatment was conducted at the Drexel Material Characterization Facility. The blood sample was treated for 30 s by e-plasma using the setup illustrated in Figure 1. After the treatment, the clot layer was gently transferred onto a silicon wafer and immediately fixed overnight in 2.5 % glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4 [12], [13], [14]. The clot layer was then washed four times in distilled water and dehydrated in a graded series of increasing ethanol concentration (30-100%) over two hours. The specimens were mounted, sputter coated with platinum palladium in a SEM coating unit for 15 s, and examined in a Philips XL30 Field Emission Environmental Scanning Electron Microscope.

III. RESULTS AND DISCUSSION

Evaluated visually, a drop of blood, with a volume of 500µl, drawn from a healthy donor and left on a stainless steel surface coagulates on its own in about 15 minutes, while a similar drop treated for 15 s by e-plasma coagulates in under 1 minute as shown in Figure 2 (a). Similarly, 0.5 ml of citrated whole blood left in a well does not coagulate on its own even when left in the open air for well over 15 minutes, while the same sample treated with DBD-plasma for 15 s exhibits immediate clot layer formation on the surface exposed to plasma discharge as shown in Figure 2 (b).

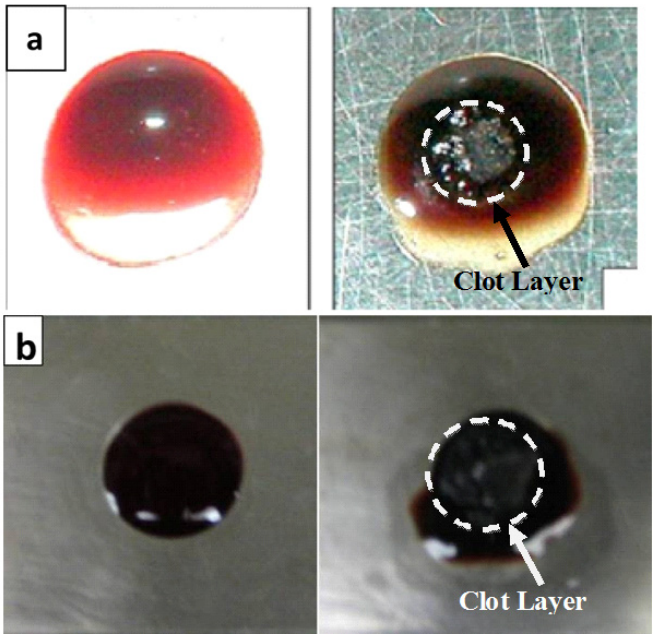


Figure 2. Coagulation of e-plasma treated non anticoagulated whole blood and citrated whole blood. (a) Non anticoagulated donor blood treated with e-plasma for 15 s exhibits immediate clot layer formation (b) Citrated whole blood treated with e-plasma for 15 s exhibits immediate clot layer formation.

To gain further insight into the structure of the clot layer of the anti-coagulated blood samples, morphological examination of the clot layer was performed by Scanning Electron Microscopy. Figure 3 (a) shows a single platelet [12], [15] over a red blood cell in untreated (control) citrated whole blood. Figure 3 (b) shows some activated, but many non-activated platelets and red blood cells in the untreated anti-coagulated whole blood. No platelet aggregation or fibrin strands are observed in these samples. On the other hand, extensive platelet activation (pseudopodia formation) and platelet aggregation was observed in the e-plasma treated

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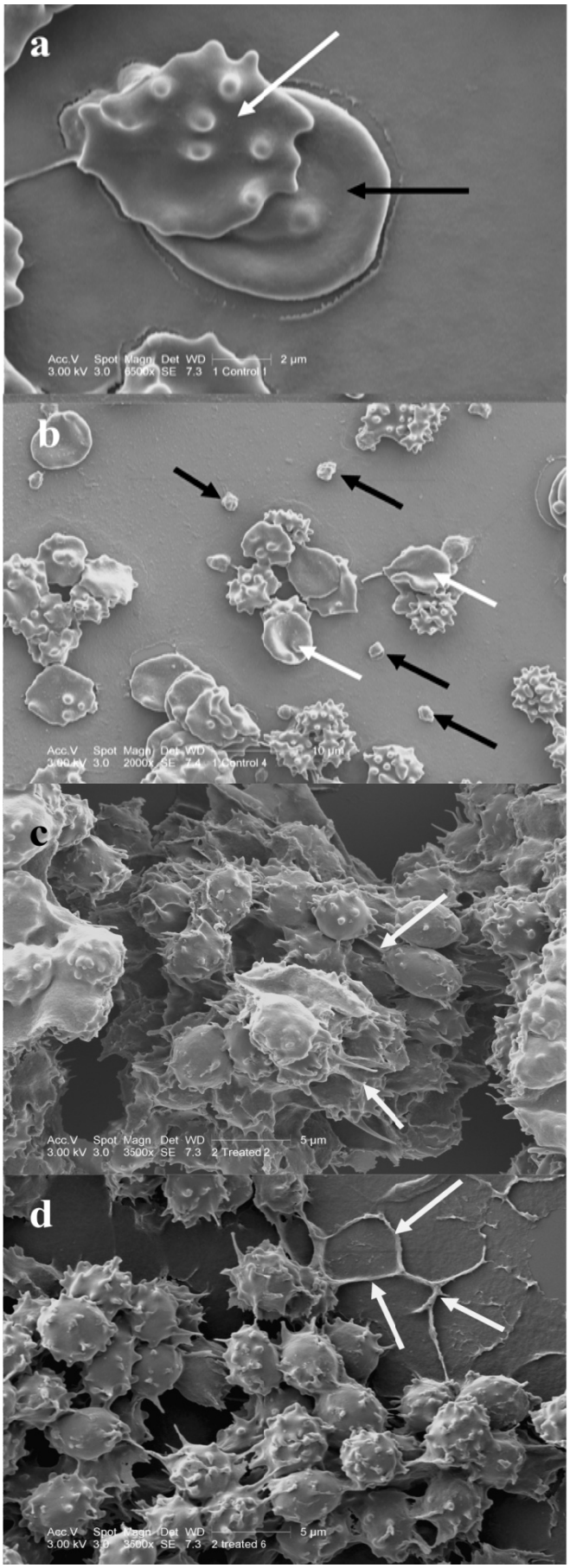


Figure 3. (a) Citrated whole blood (control) showing single activated platelet (white arrow) on a red blood cell (black arrow) (b) Citrated whole blood (control) showing many non-activated platelets (black arrows) and intact red blood cells (white arrows) (c) Citrated whole blood (treated) showing extensive platelet activation (pseudopodia formation) and platelet aggregation (white arrows) (d) Citrated whole blood (treated) showing platelet aggregation and fibrin formation (white arrows)

whole blood as evident from Figure 3 (c). Figure 3 (d) shows platelet activation, platelet aggregation and fibrin formation (white arrows) in the e-plasma treated anti-coagulated whole blood.

Natural blood coagulation is a complex process that has been studied extensively [16] and various non-thermal plasma products can affect this process at many of the steps illustrated in Figure 4 [17]. It was hypothesized previously that direct exposure to e-plasma initiates coagulation of blood through increase in concentration of Ca^{2+} [2], an important factor in the coagulation cascade as evident from Figure 4 [18], [19]. Calcium circulates in blood in several forms. 45%-50% is free ionized, 40-45% is bound to proteins, mostly albumin, and rest is

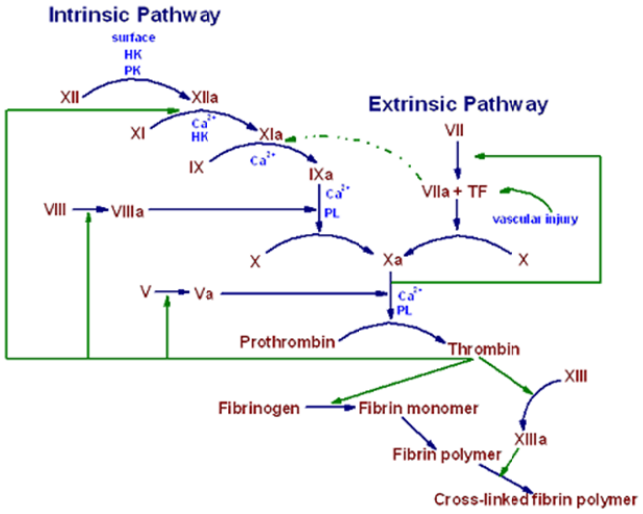


Figure 4. Simplified coagulation cascade shows the dependence of most reactions on Calcium ion Ca^{2+}

bound to anions such as bicarbonate, citrate, phosphate and lactate [20]. Bound calcium is in equilibrium with free calcium. pH has a significant effect on calcium ion binding to protein, with each unit decrease or increase in pH causing ionized calcium to change inversely by about 0.36 mmol/L [21]. It was proposed that e-plasma is effective in increase of Ca^{2+} concentration through a redox (reduction/oxidation) mechanism

$$[Ca^{2+}R^{2-}] + H^{+}_{(H_2O)} \overset{k_{Ca}|k_{-Ca}}{\longleftrightarrow} [H^{+}R^{2-}]^{-}_{(H_2O)} + Ca^{2+}_{(H_2O)}$$

provided by hydrogen ions generated through a sequence of ion molecular processes [22] (here R represents calcium-binding protein complexes, like S100A7 [23], or albumin [24], [25], [26] for example).

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We tested the validity of this hypothesis experimentally by measuring Ca^{2+} concentration in the e-plasma treated anti-coagulated whole blood using a calcium selective micro-electrode as described in the methods section. Calcium concentration was measured immediately after e-plasma treatment for 5 s, 15 s, 30 s, 60 s and 120 s. Figure 5 demonstrates that calcium concentration remains almost constant for up to 30 s of treatment and then increases very slightly for prolonged treatment times of 60 s and 120 s.

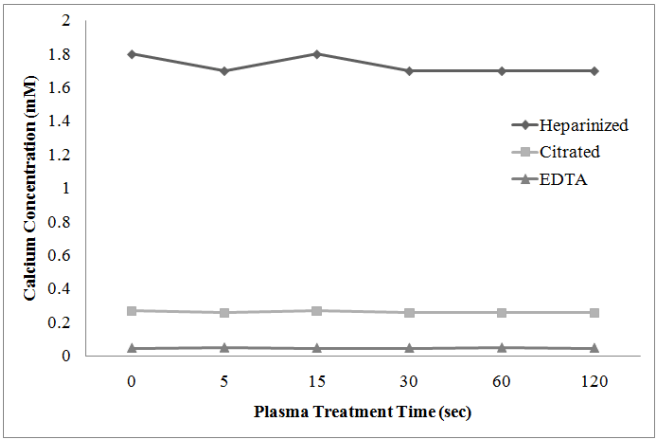


Figure 5. Calcium concentration in different anti-coagulated whole blood treated with e-plasma. There is no significant change in calcium ion concentration during the time e-plasma treated blood coagulates. (Note: average error is less than 0.01 mM)

Thus, although, e-plasma is capable of coagulating anti-coagulated blood within 15 s, no significant change occurs in calcium ion concentration during this time in discharge treated blood. Therefore, we conclude that release of Ca^{2+} may not be the mechanism for e-plasma triggered coagulation.

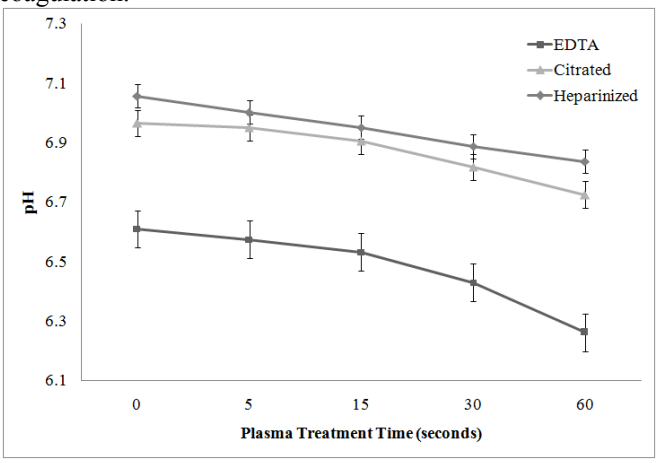


Figure 6. pH of whole blood after e-plasma treatment for different durations. pH does not change significantly in the duration in which plasma treated blood coagulates.

Changes in blood pH could also trigger blood coagulation through increase in calcium ion concentration due to redox mechanism [2]. In-vivo, the pH of blood is maintained in a very narrow range of 7.35-7.45 by various physiological processes [17]. E-plasma has been confirmed to generate a significant amount of hydrogen ions which changes pH of water and phosphate buffered saline significantly within 30 s of treatment [2]. We tested the hypothesis that the e-plasma treatment triggers coagulation through changes in the pH of blood. This was tested by measuring pH of each blood sample immediately after e-plasma treatment as described in the methods section.

Figure 6 shows that no significant change in pH occurs in the anti-coagulated blood samples during the time needed for e-plasma treated blood to coagulate.

The changes in pH obtained through e-plasma treatment are smaller than the natural variation of pH found in stored anti-coagulated blood as evident from the data plotted for the untreated samples (0 s treatment).We therefore concluded that, coagulation of blood by e-plasma treatment does not occur due to changes in pH or Ca^{2+} concentration.

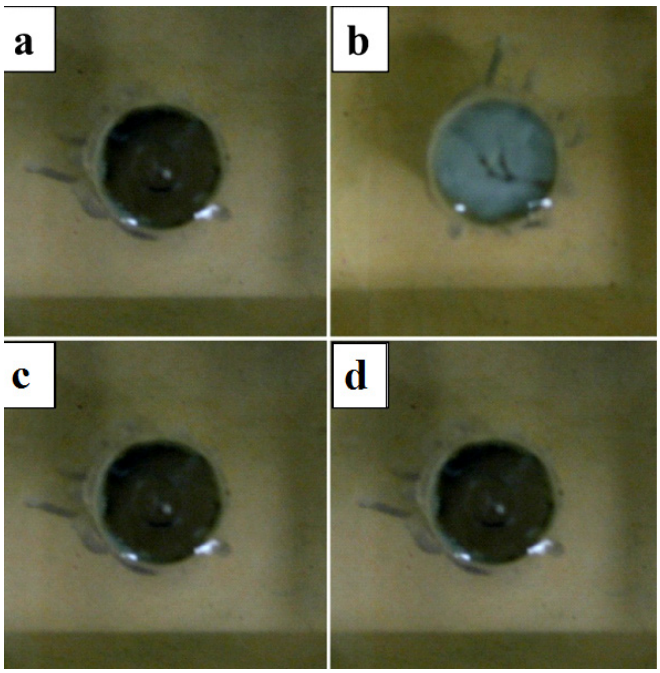


Figure 7. Treatment of buffered solution of fibrinogen (a (control), b (30 sec) and buffered solution of albumin (c (control), d (30 sec)). Figs. a-b show change in opacity of fibrinogen solution after treatment whereas Figs. c-d show no change in opacity of albumin solution.

It was previously demonstrated [2] that significant changes occur in blood plasma protein concentrations after treatment by e-plasma of samples from non anticoagulated and blood with various anti-coagulants. Direct activation of intermediate protein factors by e-plasma may be one of the mechanisms of coagulation of blood.

As shown in Figure 4, conversion of fibrinogen into cross-linked fibrin fibers is the final step in coagulation of blood. Therefore, we postulated that one of the pathways by which e-plasma treatment coagulates blood may be through direct effect on fibrinogen

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To test this hypothesis we investigated the effect of the e-plasma treatment (30 sec) on buffered fibrinogen solution (TRIS buffered saline solution, 20 mM) at physiological pH of 7.4 using the set up used for blood treatment. As compared to the untreated fibrinogen solution shown in Figure 7 (a), the opacity of the treated fibrinogen solution changes as shown in Figure 7 (b) indicating that e-plasma initiates changes in the fibrinogen solution

The above results together with the SEM images (Figure 3) showing formation of fibers suggest that one important pathway through which e-plasma treatment coagulates blood plasma is direct conversion of fibrinogen into fibrin fibers. The question arises: How specific is the effect of the e-plasma treatment on the proteins found in blood?

To test the specificity of the e-plasma treatment on blood proteins, albumin solution was tested in the same fashion as the fibrinogen solution. Albumin is an important blood protein that does not participate in the coagulation cascade, but is used as biological glue in some cases [27], [[28]. However, albumin solution prepared in the same way as the fibrinogen solution shows no visual change even after ten minutes of e-plasma treatment. (Figure 7 (c-d)).

The fact that albumin is unaffected by the e-plasma treatment indicates that this treatment can be specific in its effects on blood proteins.

IV. CONCLUSION

It has been demonstrated earlier that non-thermal plasma coagulates blood rapidly [2] and the results presented in this article indicate that non-thermal dielectric barrier discharge treatment is capable of coagulating anti-coagulated blood. This discharge appears to promote rapid blood coagulation by enhancing the natural coagulation processes. Previously it was hypothesized that direct contact non-thermal e-plasma treatment initiates blood coagulation by an increase in the concentration of Calcium ions [2], an important ion in the coagulation cascade. Experiments performed by us show no significant change in calcium concentration during the time of coagulation in discharge treated blood. E-plasma treatment does not coagulate blood due to change in pH, as we observe no significant change in pH of blood during the time of treatment in which blood coagulates. We hypothesize that e-plasma treatment may activate some of coagulation proteins. E-plasma treatment of a buffered solution of human fibrinogen results in rapid fibrinogen aggregation. Interestingly, this non-thermal plasma treatment is selective, as a similar buffered solution of human serum albumin shows no change even after a longer treatment. The results presented in this paper indicate that direct conversion of fibrinogen into fibrin may be one of the mechanisms by which non-thermal plasma initiates coagulation. Further investigations are necessary to determine other pathways of activation of coagulation by non-thermal plasma treatment.

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