

sMechanism of Blood Coagulation by Non-Equilibrium Atmospheric Pressure Dielectric Barrier Discharge Plasma

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Abstract: Mechanisms of blood coagulation by non-thermal plasma are investigated. This discharge promotes rapid blood coagulation by enhancing natural coagulation processes. Selectivity of this non-thermal discharge in treatment of various blood proteins is discussed. We demonstrate selective conversion of fibrinogen as one of the potential mechanisms by which non-thermal plasma initiates blood coagulation.

Keywords: Non-thermal plasma, Dielectric barrier discharge, Blood coagulation.

1. Introduction

Non-thermal atmospheric pressure plasma has emerged as a new promising tool in medicine. Compared to the effects of the more conventional thermal plasma [1], non-thermal plasma can be selective in its treatment because it does not burn tissue [2]. This enables a number of new medical applications including induction of apoptosis in malignant tissues [4], [8], modulation of cell attachment [3], [4], [5], [6], enhancement of wound healing, sterilization of living tissue without its damage and blood coagulation [2].

Authors have previously reported the ability of non-thermal plasma to coagulate blood [2]. Coagulation was then tested on SKH1 mice to achieve similar result. Twenty seconds of non-thermal plasma treatment was able to coagulate blood at the surface of a cut saphenous vein of a mouse as shown in Fig. 1. Here only the ability of direct non-thermal plasma treatment to coagulate blood was tested. Further investigation of ability of non-thermal plasma to hasten blood coagulation is needed and in this paper we explore a few potential mechanisms of blood coagulation due to non-thermal plasma treatment.



Fig. 1 Blood Coagulation in-vitro. (a) Saphenous vein is a major blood vessel for mice (b) If left untreated following a cut animal will bleed out (control) (c) Non-thermal plasma treatment stops the bleeding right after treatment

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. Firstly and most importantly, it will be demonstrated that direct plasma triggers natural, rather than thermally induced, coagulation processes. Secondly, it will be shown that release of calcium ions and changes of blood pH level which may be responsible for coagulation are insignificant. Instead, it will be concluded that the evidence points to selective action of direct non-thermal plasma on blood proteins involved in 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. The presence of an insulator between the electrodes prevents the build-up of high current. As a result, the non thermal plasma 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 [2], [3].

2. Materials, Methods and Experimental Setup

In this paper we investigate mechanisms of blood coagulation by non-thermal atmospheric pressure plasma using an experimental setup described by the authors [2], schematically illustrated in Fig. 2. E-plasma was generated by applying alternating polarity pulsed voltage of ~35kV 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 was used as an insulating dielectric barrier. The

discharge gap between the bottom of the quartz glass covering the copper electrode and the surface of the sample being treated was set to 2 mm. The diameter of the copper electrode employed was 2.5 cm.

For plasma treatment of 500 μ l 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. Since our interest is primarily in studying mechanisms of blood coagulation by non-thermal plasma, de-identified whole blood samples with various anticoagulants were obtained from Drexel University College of Medicine. (DUCOM) Chemistry lab. 500 μ l of 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 effect of e-plasma treatment and measure change in pH and calcium concentration after treatment. 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 (Lazar Labs PHR146XS). Similarly calcium concentration was measured after 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™).

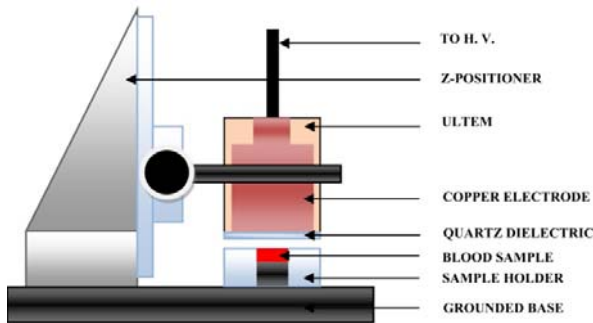


Fig. 2. Schematic of the experimental setup showing the high voltage electrode and the sample holder

To study the effect of 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 tris-hydroxymethylaminomethane (TRIS) buffered saline to obtain albumin solution 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 (37°C) TRIS buffered saline (Sigma-Aldrich, St. Louis, MO) and slowly agitated for two hours to obtain fibrinogen solution at physiological

pH of 7.4.

To determine effects of average heating due to e-plasma treatment 0.5 ml of anticoagulated whole blood was treated by covering the surface of blood by an aluminium foil. The aluminium foil served as a screen to all active elements of the e-plasma while transferring all the heat from the e-plasma discharge to the sample. This treatment was compared to treatment of anticoagulated whole blood without covering the surface.

3. Results and Discussion

Non-thermal atmospheric pressure dielectric barrier discharge plasma was experimentally verified to significantly hasten blood coagulation. Low doses of e-plasma were shown to promote and/or hasten the physiological coagulation process [2]. Visually, a drop of blood, with a volume of 0.5 ml, 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 seconds by e-plasma coagulates in under 1 minute as shown in Fig. 3 (a). 0.5 ml of anti-coagulated 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 seconds exhibits immediate clot layer formation on the surface exposed to plasma discharge as shown in Fig. 3(b).

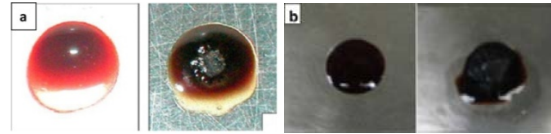


Fig. 3. (a) Coagulation of normal whole blood by e-plasma treatment, (b) Coagulation of anti-coagulated whole blood by same e-plasma treatment

Thus e-plasma can coagulate even anti-coagulated whole blood in the same time as that required for non anti-coagulated donor blood. This is interesting because anticoagulants like sodium heparin bind thrombin, in the coagulation cascade thus slowing coagulation, where as sodium citrate or ethylene diamine tetraacetic acid (EDTA), are designed to bind calcium, an important factor in the cascade, thereby, preventing coagulation altogether. Specific mechanisms by which e-plasma could coagulate blood were investigated in this paper. During physiological coagulation, many biologically complex processes take place. These processes have been studied extensively [11]. Blood coagulation is, in general, a complex process that involves platelets, various coagulation proteins, and ions. A simplified model of the coagulation cascade indicating the role of calcium ions is shown in Fig. 3. As shown in Fig. 4 most reactions in the coagulation cascade depend on calcium ion concentration [12], [13], [14]. In-vivo, platelet activation usually initiates the coagulation cascade leading to platelet aggregation and then conversion of fibrinogen into cross-linked fibrin occurs, binding platelets to form a clot. Non-thermal plasma

may coagulate blood through multiple pathways including platelet activation, activation of intermediate protein factors and increasing concentration of ionic species.

Previously it was hypothesized by the authors, 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. It was proposed that e-plasma is effective in increase of Ca^{2+} concentration through a redox (reduction/oxidation) mechanism provided by hydrogen ions generated in air plasma through a sequence of ion molecular processes [15]. We tested the validity of this hypothesis experimentally by measuring Ca^{2+} concentration in the e-plasma treated anti-coagulated whole blood using standard millivolt meter and calcium selective micro-electrode. Calcium concentration was measured immediately after plasma treatment.

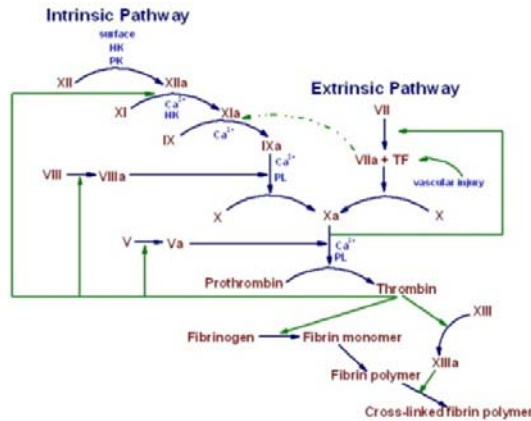


Fig. 4. Simplified coagulation cascade shows the dependence of most reactions on Calcium ion Ca^{2+}

It was seen that calcium concentration remains almost constant for prolonged treatment times of 120 s. Although, e-plasma is capable of coagulating anti-coagulated blood within 15 seconds, no significant change occurs in calcium ion concentration during the typical time of blood coagulation in discharge treated blood.

In-vivo, the pH of blood is maintained in a very narrow range of 7.35-7.45 by various physiological processes. Authors have confirmed the ability of e-plasma to generate a significant amount of hydrogen ions which changes pH of water and phosphate buffered saline significantly within 30 seconds of treatment [2]. Acidification of the biological sample being treated by plasma could be one of the mechanisms by which discharge treated blood coagulates. We tested this hypothesis by measuring pH of each blood sample immediately after e-plasma treatment using a standard pH meter and pH electrode. No significant change in pH occurs in any of the anti coagulated blood samples in the time needed for e-plasma treated blood to coagulate. The change in pH is less than the natural variation of pH found in stored anticoagulated blood. Thus coagulation of blood due to e-plasma treatment does not

occur due to change in pH or calcium ion concentration.

We explored a possibility that average heating of the sample due to e-plasma treatment could lead to coagulation of blood. To determine whether this non-thermal plasma coagulates blood due to average heating we compared the treatment of two anticoagulated whole blood samples, one covered with aluminium foil (to transfer all the heat generated by e-plasma directly to the sample) and the other uncovered.

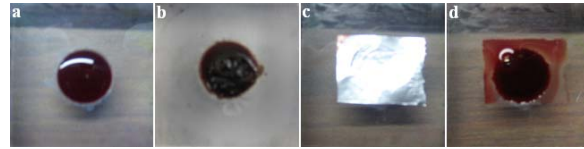


Fig. 5. (a-b) Anticoagulated whole blood (uncovered); (c-d) Anticoagulated whole blood covered with aluminium foil

We treated both samples for 30 seconds. The uncovered blood sample as shown in Fig. 5 (a-b) showed clot layer formation immediately after e-plasma treatment, while the sample covered with aluminium foil as shown Fig. 5 (c-d) did not exhibit any clot formation. The aluminium foil screens plasma to remove all active elements of plasma, except heat. Therefore we conclude that average heating is not responsible for e-plasma induced blood coagulation.

It has been demonstrated earlier that a significant change occurs in blood plasma protein concentration after treatment by e-plasma of samples from healthy donors and blood samples with various anticoagulants [2]. Activation of intermediate protein factors by e-plasma treatment may be one of the mechanisms of coagulation of blood due to e-plasma treatment. The final step in the coagulation process is the production of thrombin that converts fibrinogen into cross-linked fibrin as shown in Fig. 4. Fibrinogen is an acute phase reactant protein playing an important role in the final coagulation pathway [16]. Therefore we investigate the effect of e-plasma treatment on fibrinogen solution at physiological pH of 7.4. To eliminate the effects of change in pH, if any, due to e-plasma treatment, a buffered solution of fibrinogen was treated with non-thermal plasma. Fibrinogen dissolved in TRIS buffered saline solution was treated with e-plasma for 30 seconds. As compared to untreated fibrinogen solution shown Fig. 6 (a), post e-plasma treatment opacity of fibrinogen solution changes significantly as shown in Fig. 6 (b); indicating that e-plasma initiates changes in the fibrinogen solution. The change in opacity is an indication of polymerization taking place in the fibrinogen solution post e-plasma treatment leading to fibrin formation. Interestingly this discharge treatment is selective as a similar buffered solution of albumin shows no change even after treatment for as long as ten minutes. Fig. 6 (c-d) shows that albumin solution does not undergo

any change in opacity after plasma treatment for 30 seconds.

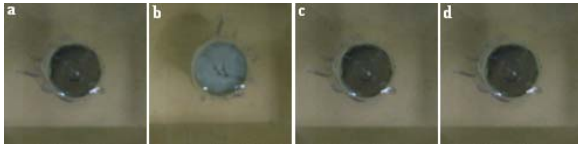


Fig. 6. Treatment of buffered solution of fibrinogen (a (control), b (30 sec)) and albumin (c (control), d (30 sec)).

We tested two types of blood proteins; proteins those take part in the coagulation cascade (for e. g. fibrinogen) and proteins those do not take part in the coagulation cascade (for e. g. albumin). E-plasma is selective in its treatment in the sense that, it influences blood coagulation proteins significantly more than proteins which do not take part in the coagulation cascade. Thus selective conversion of fibrinogen into fibrin could be one of the potential mechanisms of blood coagulation due to e-plasma treatment.

4. 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 due to an increase in the concentration of Calcium ions [2], an important ion taking part in the coagulation cascade. Experimental verification reported in this article shows no significant change in calcium concentration during the typical time of blood 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 also demonstrate that average heating is not responsible for coagulation of blood. Blood coagulation proceeds *in vivo* through a complex multi-path cascade leading to the formation of fibrin clot. E-plasma treatment may activate some of the coagulation proteins and indeed e-plasma exposure of a buffered solution of human fibrinogen for only five seconds results in a clear change of the opacity of the solution. Interestingly this non-thermal plasma treatment is selective to fibrinogen as a similar buffered solution of human serum albumin shows no change even after a longer treatment. Results presented in this paper indicate that selective conversion of fibrinogen into fibrin is one of the potential mechanisms by which non-thermal plasma initiates blood coagulation. Further investigations are needed to determine the specific mechanisms of activation of fibrinogen by non-thermal plasma treatment.

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