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Research Article

Nitroglycerin Inhibition of Thrombin-Catalyzed Gelation of Fibrinogen

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Keywords

- Nitroglycerin
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Abstract

Introduction: Nitroglycerin is widely administered intravenously. The ability of Nitroglycerin to inhibit blood coagulation by inhibiting platelet activation is well established. Previously we demonstrated that nitroglycerin could also significantly increase partial thromboplastin times.

Objective: The primary objective was to study whether the mechanism was by direct inhibition of thrombin by nitroglycerin.

Method: Time-to-gel measurements were conducted in an in vitro assay where fibrinogen solutions were converted to fibrin gels at thrombin and fibrinogen concentrations within normal physiologic ranges.

Results: Nitroglycerin directly inhibited thrombin from catalyzing fibrinogen gelation in a concentration-dependent manner that was statistically significant at nitroglycerin concentrations used for intravenous infusions.

Conclusion: The nitroglycerin concentration-dependence of the delay in time-to-gel was quantitatively consistent with theoretical predictions of the concentration-dependence of inhibitors on enzymes following Michaelis-Menten kinetics.

INTRODUCTION

Locking and flushing of lumens of central lines are essential practices for maintaining lumenal patency [1]. In order to both prevent Catheter related Bloodstream Infection and preserve patency, we previously tested an alternative lock-flush solution containing Nitroglycerin, citrate, and ethanol (NiCE) in 60 cancer patients with hematologic malignancies and at high risk for infectious and thrombotic catheter complications [2]. Primary findings from the study were that NiCE lock-flush was well tolerated and there were no CRBSIs while NiCE was administered daily. More recently, a patency analysis was completed comparing occlusion events while NiCE was administered to occlusions when saline and heparin were administered and found no difference in incidence of occlusions [3].

In vitro partial thromboplastin time (PTT) measurements showed citrate plus ethanol (CE) significantly delayed clot formation versus a saline control and that NiCE significantly extended the time-to-clot formation over CE [3]. To better elucidate the mechanism of anticoagulant enhancement of nitroglycerin, here we assessed whether nitroglycerin can directly inhibit thrombin using an in vitro model of thrombin catalyzed gelation of fibrinogen to fibrin.

METHODS

Fibrinogen Concentrate solutions were made with lyophilized fibrinogen in phosphate buffered saline (PBS), Thrombin Concentrate solutions were made with lyophilized thrombin in PBS, Calcium Chloride Concentrate solution was made with deionized water and Dextrose Concentrate was prepared with deionized water (all reagents purchased from Sigma Aldrich, St. Louis, MO). Gel solutions were made by combining the diluted Fibrinogen solution with Calcium Chloride solution and warming to 37°C. The resulting solution was mixed with Thrombin solution and either Nitroglycerin+Dextrose Concentrate (400 micrograms/mL Baxter Deerfield, IL) or Dextrose solution warmed to 37°C in a vial. Since the Nitroglycerin Concentrate contained Dextrose, Dextrose only served as the control for isolating the impact of nitroglycerin on thrombin-catalyzed fibrinogen gelation. The final concentrations of the mixtures had Thrombin concentrations of 0.1 or 0.05 Units/mL, 3.4 or 1.7 mg/mL concentrations of Fibrinogen and were 2.5 mM in calcium chloride. Nitroglycerin+Dextrose concentrations in the gel solutions where gelation occurred ranged from 0 to 120 micrograms/mL (nitroglycerin) and 0 to 1.5% Dextrose. Dextrose-only control gel solutions had corresponding dextrose concentrations ranging from 0 to 1.5%. The vial was inverted

every 20 seconds until gelation occurred as detected from loss of fluidity of the vial contents. The time to gelation (in seconds) was noted. Three replicate gel time measurements were performed for each fibrinogen, thrombin, nitroglycerin+dextrose and dextrose-only combination. 2-sided statistical t-tests were performed using Microsoft Excel and p values of less than or equal to 0.05 were considered statistically significant.

RESULTS

Figure 1 presents a plot of mean gelation times measured for 3.4 mg/mL Fibrinogen systems (0.1 Units/mL Thrombin with nitroglycerin+dextrose or control (dextrose-only) and 0.05 Units/mL Thrombin with nitroglycerin+dextrose or control (dextrose-only).

Table 1 presents computed mean gelation time differences (difference in gelation time between nitroglycerin and dextrose versus control [dextrose-only]) over a range of different nitroglycerin concentrations for 0.1 and 0.05 Units/mL thrombin and 3.4 mg/mL fibrinogen. The differences for 3.4 mg/mL fibrinogen were statistically significant at nitroglycerin concentrations of 7.5 micrograms/mL and 30 micrograms/mL and above (depending on Thrombin concentration). The dashed vertical lines in Figure 1 (at thrombin concentrations of 0.1 or 0.05 Units/mL) present the threshold nitroglycerin concentrations at which inhibition is statistically significant. All values to the right of the vertical dashed line represent statistically significant inhibition of thrombin- catalyzed fibrinogen gelation by nitroglycerin. Figure 2 plots mean gelation times measured for 1.7 mg/mL Fibrinogen systems (0.1 Units/mL Thrombin with nitroglycerin+dextrose or control (dextrose-only) and 0.05 Units/mL Thrombin with nitroglycerin+dextrose or control (dextrose- only)). Table 1 presents computed mean gelation time differences (difference in gelation time between nitroglycerin and dextrose versus control [dextrose-only]) over a range of different nitroglycerin concentrations for 0.1 and 0.05 Units/mL thrombin and 1.7 mg/mL fibrinogen. The differences for 1.7 mg/mL fibrinogen were statistically significant at nitroglycerin concentrations of 15 micrograms/mL and above and 7.5 micrograms/mL and above (depending on thrombin concentration). The dashed vertical line in Figure 2 (at thrombin concentrations of 0.1 or 0.05 Units/mL) presents the threshold nitroglycerin concentration at which inhibition is statistically significant. All values to the right of the vertical dashed line represent statistically significant inhibition of thrombin catalyzed fibrinogen gelation by nitroglycerin.

DISCUSSION

Our results indicate that nitroglycerin inhibits thrombin catalyzed gelation of fibrinogen in a concentration dependent manner. At the higher fibrinogen concentration (3.4 mg/mL), inhibition became significant at a nitroglycerin concentration of 7.5 or 30 micrograms/mL (lower threshold for higher thrombin concentration) and at the lower fibrinogen concentration (1.7 mg/mL), inhibition became significant at a nitroglycerin concentration of 7.5 or 15 micrograms/mL (lower threshold for lower thrombin concentration). Similarly, at the higher thrombin concentration tested, inhibition became significant at a nitroglycerin concentration of 7.5 or 15 micrograms/mL and at

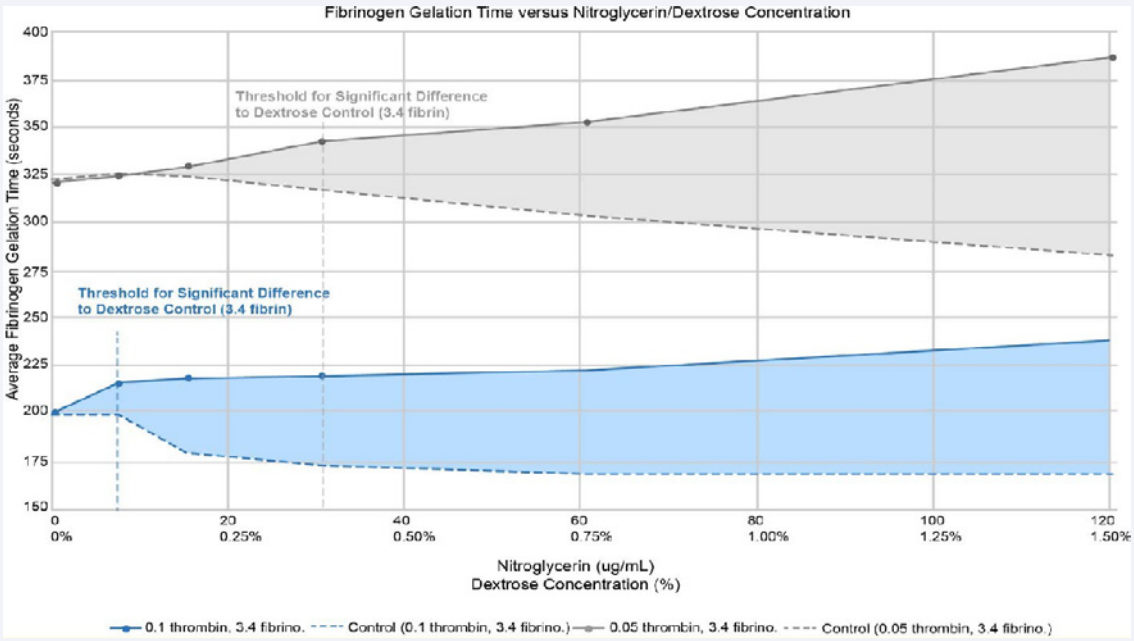

Figure 1 Gelation Time as a function of Nitroglycerin/Dextrose Concentration (3.4 mg/mL Fibrinogen Concentration with 0.1 Units/mL and 0.05 Units/mL Thrombin Concentrations)

Gelation time as a function of Nitroglycerin concentration is plotted for 3.4 mg/mL Fibrinogen concentration. The solid and dashed line pairs are gelation times for nitroglycerin + dextrose (solid) and for dextrose-only control (dashed). The top pair of lines is for 0.05 Units/mL Thrombin and the bottom pair for 0.1 Units/mL. The inhibitory increase in gelation time for each pair is reflected by the increase in shaded area as nitroglycerin concentration is increased. The Nitroglycerin concentration thresholds for statistical significance are displayed. At a nitroglycerin concentration of 240 µg/ml Thrombin catalyzed gelation of Fibrinogen was completely inhibited.

Table 1: Gel Time difference between Nitroglycerin+Dextrose Solutions and Dextrose-only Controls.

Fibrinogen Concentration (ug/mL)	Thrombin Concentration (Units/mL)	Computed gel time difference (Δ t in s) between Nitroglycerin solutions and Dextrose only controls for different Nitroglycerin concentrations (ug/mL)						Calculated correlation coefficient (Δ t versus nitroglycerin concentration)
		0 ug/m	7.5 ug/mL	15 ug/mL	30 ug/mL	60 ug/mL	120 ug/mL	
3.4	0.1	2 s	17 s *	40 s *	46 s *	54 s *	71 s *	**0.875**
1.7	0.1	-2 s	5 s	10 s *	18 s *	28 s *	54 s *	**0.993**
3.4	0.05	-2 s	-1 s	5 s	26 s*	50 s *	104 s *	**0.997**
1.7	0.05	13 s	34 s*	37 s *	47 s *	53 s *	123 s *	**0.970**

Tabulation of mean gel time differences between measured nitroglycerin+dextrose versus dextrose-only solution gelation times at the same fibrinogen and thrombin concentrations.
*Denotes difference was statistically significant ($p \leq 0.05$). Correlation coefficient was calculated from linear regression analysis on Δ r versus nitroglycerin concentration as described in the discussion.

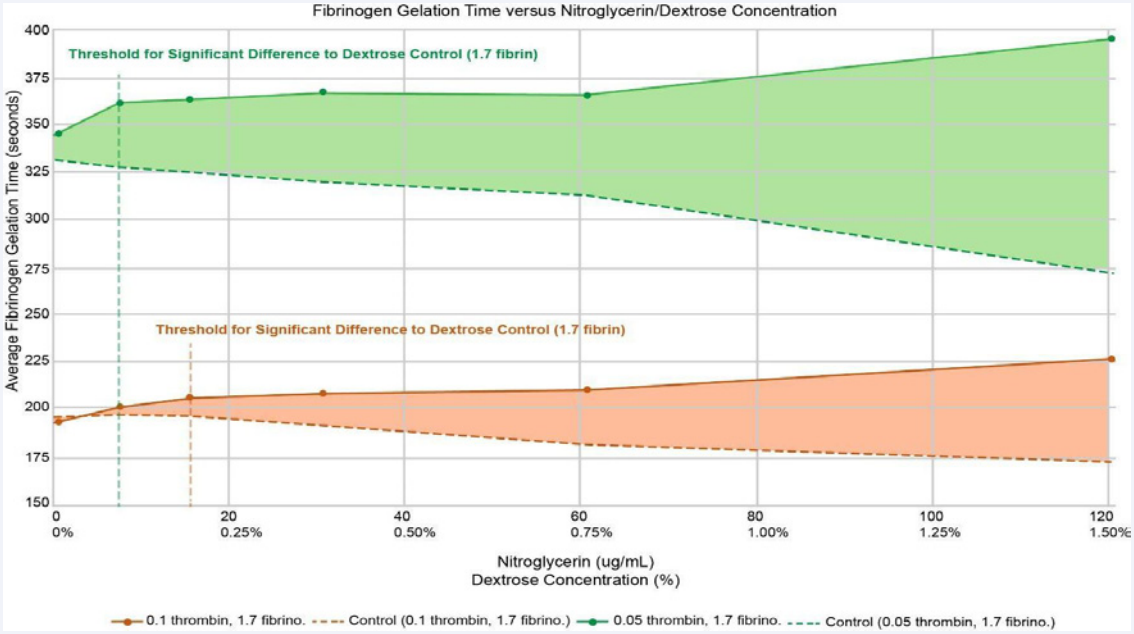

Figure 2 Gelation Time as a function of Nitroglycerin/Dextrose Concentration (1.7 mg/mL Fibrinogen Concentration with 0.1 Units/mL and 0.05 Units/mL Thrombin Concentrations
Gelation time as a function of Nitroglycerin concentration is plotted for 1.7 mg/mL Fibrinogen concentration. The solid and dashed line pairs are gelation times for nitroglycerin + dextrose (solid) and for dextrose-only control (dashed). The top pair of lines is for 0.05 Units/mL Thrombin and the bottom pair for 0.1 Units/mL. The inhibitory increase in gelation time for each pair is reflected by the increase in shaded area as nitroglycerin concentration is increased. The Nitroglycerin concentration thresholds for statistical significance are displayed. At a Nitroglycerin concentration of 240 μg/ml Thrombin catalyzed gelation of Fibrinogen was completely inhibited.

the lower thrombin concentration, inhibition became significant at a nitroglycerin concentration on 7.5 or 30 micrograms/mL. Since nitroglycerin was formulated with dextrose, increasing the concentration of nitroglycerin necessarily increased the concentration of dextrose. The relatively high concentrations of dextrose as well as the sensitivity of the gel-time detection method may have impacted the quantitative nitroglycerin concentration limit where inhibition became significant, and these limits well might have been lower had no dextrose been present or more sensitive gel point detection methodology been employed.

Without nitroglycerin present (i.e., the dextrose-only control), increasing the concentration of dextrose appeared to accelerate enzymatic conversion of fibrinogen (reduced the time to gel). The acceleration is weak in that increasing dextrose from 0 to 1.5% reduced gelation time by about 10-20% depending on thrombin and fibrinogen concentrations.

Conversely, increasing the concentration of nitroglycerin and dextrose together inhibited enzymatic conversion (progressively increased the time to gel). The magnitude of the inhibition (time to gel with nitroglycerin and dextrose minus time to gel with same amount of dextrose only = Δt) was significantly dependent on the nitroglycerin concentration. The magnitude of inhibition is graphically presented in Figures 1 and 2 as the vertical gap between the nitroglycerin and dextrose time to gel curve and the dextrose only time to gel curve. The gap (Δt) monotonically increases as the nitroglycerin concentration increases.

The concentrations of fibrinogen tested were within the normal physiologic range [4]. The thrombin concentrations tested were comparable to physiologically reported proteinolytic thrombin levels (0.1 – 0.2 IU/mL)[5] (Stief, Thomas. (2016). Re: What are the normal concentrations of thrombin in human serum and blood?. Retrieved from: https://www.researchgate.net/post/What-are-the-normal-concentrations-of-thrombin-in-human-serum-and blood/57b17fe0cbd5c2a36977b644/citation/download.) The formation of gels as a result of thrombin catalyzed conversion of fibrinogen has been reviewed. It was reported that the gel point occurs when 15-20% of the fibrinogen has been converted to fibrin [4]. In the absence of dextrose and nitroglycerin, fibrinogen did gel in the presence of thrombin as expected. Figure 1 and 2 show the time to gel was strongly dependent on the thrombin concentration at the fibrinogen concentrations tested (gelled much faster with higher thrombin concentration) but only weakly dependent on the fibrinogen concentration at the thrombin concentrations tested.

The times to gel we measured with zero dextrose or nitroglycerin were consistent with the times to gel reported by Kita et al., using more sensitive light scattering methodology [6].

Thrombin catalyzed conversion of fibrinogen has been shown to follow Michaelis- Menten enzyme kinetics [7,8]. Bezerra et al., presented integrated Michaelis-Menten enzyme kinetics equations in the presence of inhibitors providing a time dependence of substrate consumption (fibrinogen in our system) as a function of inhibitor concentration. Their eqs. 6-8 present linear dependences of time on inhibitor concentration (Po) [9]. Consequently, in our experiment, if Nitroglycerin inhibition followed classical enzyme inhibitor kinetics, the difference in time to gel for nitroglycerin and dextrose versus the time to gel for the dextrose- only control (Δt) should be linearly dependent on nitroglycerin concentration. The differences in time to gel (Δt) are tabulated in Table 1 for the different Fibrinogen and Thrombin concentrations tested. Linear regression was performed on Δ t versus nitroglycerin concentration and the correlation coefficient for the magnitude of inhibition as a function of nitroglycerin concentration was calculated [10]. A correlation coefficient of unity (1) indicates a mathematically perfect linear correlation relationship. The values were nearly unity for low Thrombin concentrations as well as for the low Fibrinogen concentrations at the high thrombin concentration. The high Fibrinogen and high Thrombin concentration system still gave a reasonably linear value of 0.875. These correlations for nitroglycerin concentration dependence of inhibition of fibrinogen gelation kinetics concur with theoretical predictions from Michaelis- Menten kinetics.

In conclusion, our experiments have phenomenologically shown that nitroglycerin directly inhibits thrombin from catalyzing formation of fibrin gels from fibrinogen solutions in a concentration dependent manner quantitatively consistent with enzyme kinetic theoretical predictions. Specific inhibitory binding sites for nitroglycerin on thrombin remain to be elucidated in future studies.

STATEMENT OF CONFLICTS OF INTEREST

Drs. I Raad and J. Rosenblatt are inventors of the Nitroglycerin, Citrate, and Ethanol antimicrobial catheter lock solution technology which is owned by the University of Texas MD Anderson Cancer Center (UTMDACC) and has been licensed to Novel Anti-Infective Technologies, LLC in which UTMDACC, Dr. Raad and Dr. Rosenblatt are shareholders.

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