

Preparation, characterization and in vitro thrombolytic activity of a novel streptokinase foam

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Published online: 4 March 2014
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Abstract Vascular thrombosis is a potentially fatal disease. Thrombolysis represents an efficient therapeutic option, although it still presents intrinsic bleeding risks. In order to minimize this problem, intra-thrombus injections, alone or associated with some kind of mechanical thrombectomy, have been used. In this work, a new approach to thrombolysis is presented, where the preparation, characterization and in vitro thrombolytic activity of a novel streptokinase foam are reported. Foams were prepared by mixing albumin solution with CO₂ at different volume ratios. Foam stability and apparent viscosity were the parameters used to characterize the foams. The volume ratio between CO₂ and albumin solution that yielded the samples with the best properties was used to prepare the thrombolytic foams, where streptokinase was used as the thrombolytic agent. The thrombolytic effect of this foam was assessed in vitro by delivering it intra-thrombus and the results were compared with those of the foam without streptokinase as well as those of a regular streptokinase solution. Both foam stability and viscosity increased as the ratio of CO₂:albumin solution increased and the 3:1 ratio was used to incorporate streptokinase. The in vitro thrombolytic activity study revealed that the streptokinase

foam caused a 46.6 % of thrombus lysis after 30 min of experiment against 21 and 31 % of those of the foam without streptokinase and the regular streptokinase liquid solution, respectively. Thus, the use of CO₂:albumin foam enhanced the in vitro thrombolytic effect of streptokinase, which indicates its potential as a novel vehicle for carrying and delivering streptokinase to targeted thrombi.

Keywords Thrombolytic foam · Streptokinase · Thrombolysis · Albumin

Introduction

Although considered an important physiological mechanism, thrombosis is responsible for triggering several cardiac events such as myocardial infarction, ischemic cerebrovascular accident, systemic arterial embolism, deep venous thrombosis and acute pulmonary embolism [1–3].

The prevention of thrombus formation involves lifestyle changes, such as eating a healthy balanced diet, doing regular exercises, giving up smoking, controlling stress and keeping optimal glucose and lipid levels [4]. Besides, early detection of prothrombotic states may contribute to thrombosis prevention [5]. On the other hand, drug prophylaxis of thrombotic events aims to reduce the occurrence of the clotting cascade, which is responsible for the thrombus formation. Heparins with several molecular weights, coumadin, and antiplatelet agents have been extensively used for thrombosis treatment and prophylaxis [6, 7].

Although the use of these drugs offers a clinically useful therapy, they often fail to re-establish the normal blood flow, leading to permanent occlusion of the vessel. Thus, several thrombolytic drugs have been used in an attempt to rapidly dissolve the thrombus in a selective and safe way. However,

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the use of thrombolytics can potentially lead to complications, especially if administered systemically [7]. Due to the use of higher doses, the systemic administration of thrombolytic agents is associated with a higher incidence of systemic side effects [8, 9]. It is, therefore, vital to develop new strategies to deliver thrombolytic drugs using techniques that achieve higher local intra-thrombus concentrations of the drug [10–12]. In fact, intra-thrombus infusion of thrombolytic agents has proven to be a highly successful means of restoring vascular function [11, 13, 14].

Conventionally, thrombolytic agents are administered as liquid solutions, where the drug's lyophilized powder is reconstituted using an appropriate vehicle/diluent (e.g. water for injection or saline solution 0.9 %) prior to its infusion. However, intra-thrombus administration of thrombolytic solutions has been associated with its premature wash-out from the site of application as the blood flow is restored, which decreases the contact time between the drug and the thrombus, requiring repeated infusions [15, 16].

Therefore, the incorporation of the thrombolytic agent into a vehicle that would decrease its removal from the site of application and hence increase its contact time with the targeted thrombus seems to be a reasonable alternative to the commonly used liquid diluents/vehicles. For instance, the use of foams for sclerotherapy has shown to be far more effective than liquid sclerotherapy [17, 18]. In this case, the increased efficacy of foams has been attributed to a better displacement of the blood from the treated vein and to an increased contact time between the sclerosant and the venous endothelium [19]. Taking this into consideration, we decided to use the same approach to incorporate a thrombolytic agent into a biocompatible foam and investigate its effectiveness in comparison to a conventional liquid thrombolytic solution.

Herein, we discuss the preparation, characterization and *in vitro* activity of a novel thrombolytic foam, which was prepared by mixing human albumin solution with carbon dioxide at different volume ratios, where the sample that showed the best properties was used as a vehicle for the thrombolytic agent (streptokinase). The thrombolytic effect of this foam was assessed *in vitro* by delivering it intra-thrombus using a multi-perforated catheter. The results were compared to those of a regular streptokinase liquid solution and a foam without streptokinase delivered in the same way.

Materials and methods

Preparation of the foams

The foams were prepared according to the method described by Tessari et al. [20] where two 10 mL glass syringes with a three-way tap were used. After connecting both

Table 1 Composition of the foam

Volume of CO ₂ (mL)	Volume of albumin solution (mL)	Ratio of CO ₂ :albumin solution (v:v)
1	3	1:3
1.5	2.5	1.5:2.5
2	2	2:2
2.5	1.5	2.5:1.5
3	1	3:1
3.5	0.5	3.5:0.5

syringes to the three-way tap, a predetermined volume of human serum albumin solution 20 % (Baxter Healthcare Corporation, USA) and CO₂ were withdrawn according to specific volume ratios (Table 1). CO₂ and albumin solution were mixed through 20 passages between the two syringes using the stopcock as a connector. The obtained 4 mL of foam was immediately characterized. In order to prepare the thrombolytic foams, streptokinase (Solustrep, 1,500,000 U, Bergamo, Brazil) was previously dissolved in the albumin solution. This solution was then mixed with CO₂ and the procedure was followed according to the aforementioned steps. The final streptokinase concentration on the foam was 100,000 U/mL.

Characterization of the foams

Foam stability (FS) and apparent viscosity were the parameters used to characterize the foams. The cylinder method was used to determine the FS, as described by Arzhavitina and Steckel [21]. Briefly, 4 mL of the foam was discharged into a 10 mL glass cylinder right after its preparation. After 30 min, the liquid was partially separated due to liquid drainage and the volume was then recorded. FS was calculated as:

$$FS = \frac{V_{L(30\text{min})}}{V_{F(\text{initial})}} \times 100$$

where $V_{L(30\text{ min})}$ and $V_{F(\text{initial})}$ are the volume of liquid after 30 min and the initial volume of foam (~4 mL), respectively.

Apparent viscosity of the foams was determined at room temperature in a cone and plate Brookfield viscometer, model DV-III ULTRA (Brookfield Engineering Laboratories, Middleboro, MA, USA), using the software Rheocalc V3.3 Build 49-1. After preparing the foam, 1 mL of the sample was placed in the cone with a CP-52 spindle and submitted to shear rates (the ratio of flow speed and a characteristic length) in the range of 40–240 (s⁻¹), where the apparent viscosity was determined according to the shear rate.

Thrombus generation

Venous blood (200 mL), donated by a healthy volunteer, was used for the generation of all the thrombi used in this study, as well as for filling the tubes. 10 mL of blood was transferred to a centrifuge tube followed by the addition of 2 mL of calcium gluconate for inducing thrombus formation. After incubation for 2 h at 37 °C, the obtained ~7 cm long thrombus was removed from the centrifuge tube, rinsed with 0.9 % saline solution and carefully wiped off with paper towel before weighing.

Assessment of the thrombolytic effect

Prior to starting the experiment, the tip of a 5-F pulse-spray catheter (Cook Medical Inc. Bloomington, IN, USA) was occluded with silicone and then introduced longitudinally into the thrombus. After ensuring that the entire perforated segment of the catheter was located within the thrombus, 1 mL of each sample was administered. Next, the treated thrombus was transferred to the original centrifuge tube, where 5 mL of fresh blood was added. This experiment was divided into three groups: the group treated with the streptokinase foam and the two control groups comprising the one treated with foam without streptokinase and another one treated with regular streptokinase solution, each with five replicates. The concentration of streptokinase was the same for both foam and liquid solution. In addition, the volume injected into the thrombus was exactly the same for all three groups. In order to avoid variations in the infusion pressures, the samples were infused through a Fountain Infusion System® (Merit Medical Systems Inc., UT, USA), which allowed the release of the sample with a single hand motion.

Thrombolytic therapy was performed in a thermostated bath at 37 °C during 30 min. Once the experiment was terminated the excess of fluid was blotted from the sample's surface with a filter paper and the residual thrombus was then weighted (W_{30}) to observe the difference in weight after thrombus disruption. Difference obtained in weight taken before and after the thrombolytic therapy was expressed as percentage of thrombus lysis, as shown in the following equation:

$$\% \text{ lysis} = \frac{W_0 - W_{30}}{W_0} \times 100$$

where W_0 and W_{30} are the weight of the thrombus before and after 30 min of treatment, respectively. The experiments were performed in five replicates with all three groups.

Statistical analysis

The non-parametric Kruskal–Wallis test was used to test the null hypothesis that the three groups are from the same population and the Wilcoxon–Mann–Whitney rank sum test for post hoc pairwise comparison of groups, with p -values adjusted for multiple comparisons with the Bonferroni correction. Data analysis was performed using the software Stata release 11 (Stata Corp., College Station, TX, USA).

The power of the study design was calculated as follows: assuming a standard deviation of the study variable of 6.5 and an alpha error of 0.025, the sample size of this study affords a power of 58 % to detect a 50 % increase in the mean of the study variable.

Results

The preparation of the foams through the method of Tessari, using CO₂ and albumin solution as the gas and liquid phases, respectively, seemed to be easy and straightforward. All the ratios between the gas and the liquid were able to produce foams that presented a creamy consistency. The stability of the foams according to different volume ratios of CO₂ and albumin is presented on Fig. 1, where a linear relationship ($R^2 = 0.999$) was obtained. The 3:1 ratio of CO₂ to albumin solution was found to produce the most stable foam, where 72.5 % ± 0.7 of its initial volume remained after 30 min of experiment.

In order to access the influence of these ratios on the viscosity of the foams, apparent viscosity of each sample

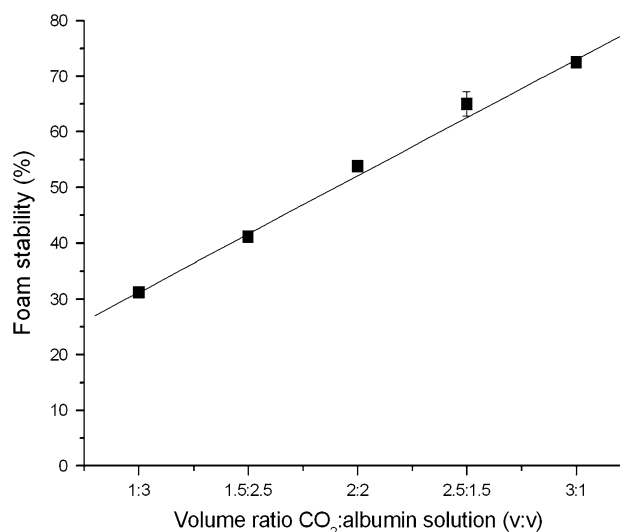


Fig. 1 Influence of the volume ratio of CO₂:albumin solution on the stability of the foams. $n = 3$; error bar = standard deviation

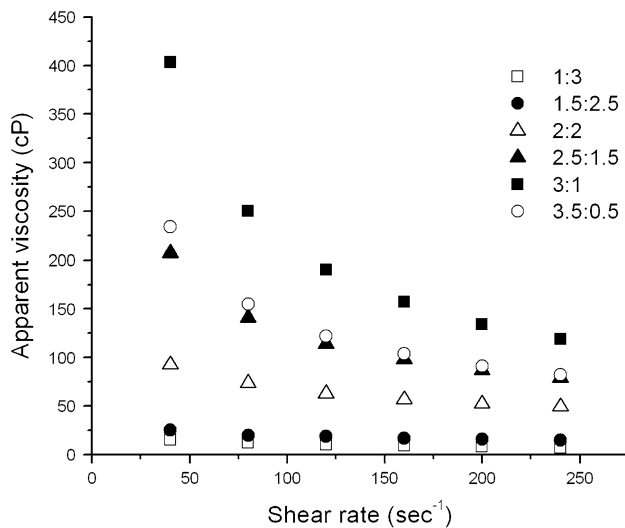


Fig. 2 Influence of the volume ratio of CO₂:albumin solution on the apparent viscosity of the foams, determined in the shear rate range of 40–240 s⁻¹

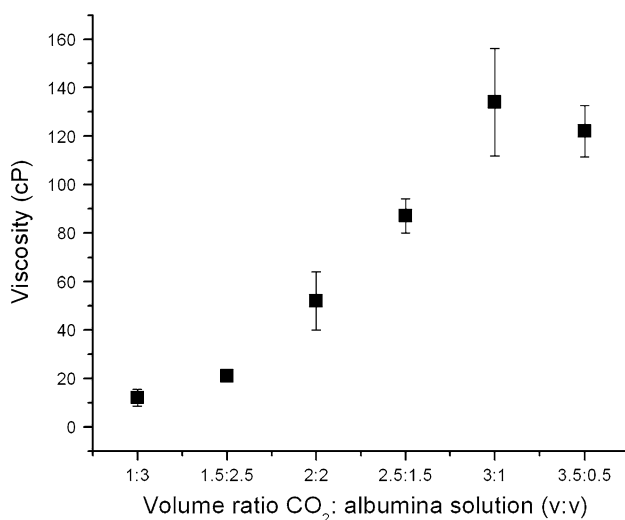


Fig. 3 Influence of the CO₂:albumin solution ratio on the apparent viscosity (determined at a shear rate of 120 s⁻¹) of the foams. $n = 3$; error bar = standard deviation

was determined within a shear rate range of 40–240 s⁻¹ (Fig. 2). As the shear rate increased the apparent viscosity of all the foams decreased, which characterizes a non-Newtonian type of behavior (a fluid with viscous behavior that depends on the actual speed).

Similarly to the stability results, the apparent viscosity of the foams increased with the increase in the CO₂:albumin solution ratio, as shown in Fig. 3. However, ratios of CO₂:albumin solution above 3:1 did not produce foams with higher viscosities. Therefore, the ratio of 3:1 was chosen to prepare the foams that were used to incorporate

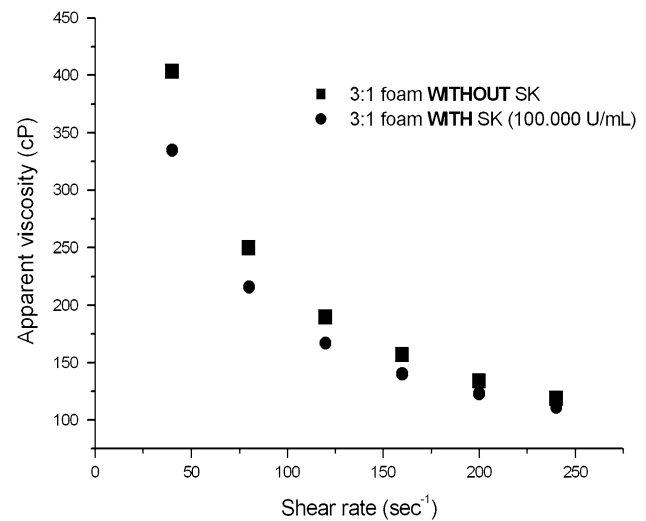


Fig. 4 Influence of the presence of streptokinase at a concentration of 100,000 U/mL in the viscosity of the obtained foams. This study was done by comparing the apparent viscosities of the thrombolytic foam (containing streptokinase) with those of foams without streptokinase

streptokinase. The thrombolytic foams were prepared by previously dissolving streptokinase in the albumin solution. Once a clear solution was obtained, it was mixed with the appropriate volume of CO₂, as previously described. In order to verify the influence of streptokinase in the viscosity of the foams, a thrombolytic foam (3:1 of CO₂:albumin solution) was prepared with 100,000 U of streptokinase per mL of foam, which corresponded to the concentration used in our in vitro study. As shown in Fig. 4, the incorporation of streptokinase had a very small effect on the viscosity of the foam, where this parameter was slightly decreased (<15 %) with the addition of this drug.

Finally, the in vitro thrombolytic activity study revealed that the use of streptokinase in saline solution and the foam without streptokinase (control groups) showed 31 % ± 2.8 and 21 % ± 7.0 of thrombus lysis, respectively, whereas the novel streptokinase foam showed 46.6 % ± 10.5 (Fig. 5). According to the Kruskal–Wallis nonparametric test, the comparison of the average of the thrombolytic activity between the three groups yielded a p value of 0.0019, whereas the comparison between each of two groups (Wilcoxon–Mann–Whitney test) yielded a p value (adjusted for multiple comparisons) of 0.027 in all three comparisons.

Discussion

Methods of delivering thrombolytic agents include systemic infusion and intra-thrombus administration, the latter being called catheter-directed therapy [20, 22]. Administration of thrombolytic agents into thrombus has become

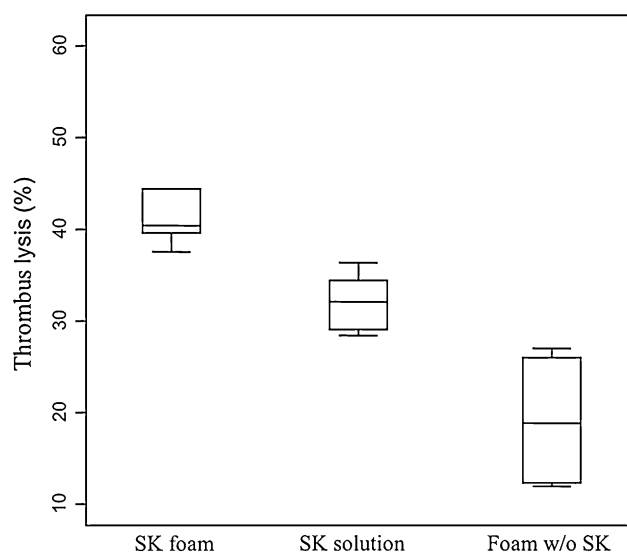


Fig. 5 Box plot diagram of the thrombolytic effect (represented by the percentage of thrombus lysis) of the novel streptokinase foam in comparison with those of the streptokinase liquid solution and the foam without drug. $n = 3$; error bar = standard deviation

accepted as the best approach to establish the blood flow in partially occluded arteries and veins [11, 22–24]. Among the three commonly used thrombolytic agents: streptokinase, urokinase and recombinant tissue plasminogen activator (rt-PA) [25], we decided to use streptokinase in our study. In Brazil, streptokinase is widely used due to its lower cost, as compared to the other thrombolytic drugs. In fact, Ouriel et al. [26] have done a comparison of these three agents in an in vitro model of venous thrombolysis and according to these authors, when expressing the efficacy of thrombolysis as a function of the cost of the pharmaceutical agent, streptokinase was the most cost-effective thrombolytic drug. In addition, other authors have reported a lower incidence of bleeding complications related to streptokinase when compared to rt-PA [14, 27–29]. However, the short contact time between the thrombolytic agent and the thrombus, due to its premature wash-out as the blood flow is restored, requires repeated infusions of the thrombolytic liquid solution, which can increase the risks of systemic side effects and other complications [15, 16].

Since a better displacement of the blood and a more effective contact with the venous endothelium are achieved with sclerosant foams rather than liquids [15–18], we thought that the same approach could be used to improve the thrombolytic effect of streptokinase. In this study, streptokinase foams were prepared and its thrombolytic activity was compared to those of a conventional streptokinase liquid solution and a foam without streptokinase.

For preparing the foams, CO_2 and human albumin solution were used as the gas and liquid phases, respectively, as both

are biocompatible and low cost components [22, 30–32]. In addition, it has been demonstrated that foams created from CO_2 causes less bubble-related side effects than foams prepared from room air [32, 33]. Although albumin microbubbles has been previously used to dissolve thrombus [34–36], its thrombolytic effect was studied only in association with ultrasound. Besides, urokinase and tPA were the only thrombolytic agents used in these studies. Thus, to the best of our knowledge, no study has been reported about the use of albumin solution and CO_2 to prepare thrombolytic foams using streptokinase as the thrombolytic agent. In this study, prior to preparing the thrombolytic foams, we decided to determine which ratio between CO_2 and albumin solution would yield the most stable and viscous foam. The results showed that both the stability and the apparent viscosity of the foams increased with the increase in the volume ratio of CO_2 :albumin solution, which means that both parameters increased as the concentration of albumin decreased. According to Arzhavitina and Steckel [21], foams with higher gas volume fractions are more stable, which is reflected as a delayed liquid drainage. In our study, the CO_2 :albumin solution volume ratio of 3:1 produced the most stable and viscous foam. Therefore, this ratio was used to incorporate streptokinase and to prepare the thrombolytic foams.

In order to study the thrombolytic activity of these foams, an in vitro experiment was performed where the sample was administered intra-thrombus using a multi-perforated catheter. The dose of streptokinase used in this study was 100,000 U/mL of foam/liquid, administered intra-thrombus through a single injection of 1 mL, where the thrombolytic activity was determined after 30 min of experiment. Streptokinase has a half-life of 20–30 min [28, 37] and since a single injection was used in this study, we wanted to make sure that the influence of the vehicle on the thrombolytic activity of streptokinase was accessed within the timeframe where this drug was fully active. Therefore, the dose of streptokinase was adjusted in such a way that a reasonable amount of thrombus would be dissolved in no longer than 30 min.

Our results showed that, although the streptokinase dose was much lower than that of a typical systemic bolus administration of a streptokinase solution for thrombolytic therapy in myocardial infarction (1,500,000 UI [37, 38]), 46.6 % $\pm$ 10.5 of the thrombus was dissolved after injecting 1 mL of the streptokinase foam, which was statistically higher than those of 1 mL of streptokinase liquid solution at the same drug concentration and 1 mL of the 3:1 CO_2 :albumin foam without streptokinase (control groups). Therefore, the thrombus lysis was more efficient when the thrombolytic agent was incorporated into the foam and since the concentration of streptokinase, the volume administered, the pressure of the infusion and the duration of the experiment were exactly the same for all

three groups, we assume that the difference in the thrombolytic activity between these groups is attributed to the vehicle (foam vs liquid) as well as to the presence of streptokinase (foam with streptokinase vs foam without streptokinase).

Thus, the foam prepared from a mixture of CO₂ and albumin solution in a volume ratio of 3:1 seems to be promising as a vehicle for carrying and delivering streptokinase to the targeted thrombus. Since it is prepared out of two biocompatible and inert materials (human albumin and CO₂) and considering that it is administered in such small quantities (around 1 mL), we strongly believe that this foam has a very low potential to cause side-effects. Other factors that contribute to its safety include: the low dose of streptokinase and its intra-thrombus administration. In vivo studies using animal models will need to be performed to determine the suitability of this novel streptokinase foam as a new candidate for a thrombolytic therapeutic agent.

Conflict of interest The authors have a patent pending related to this work (patent pending with INPI/BRAZIL – PCT INTERNATIONAL APPLICATION).

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