Original article

Effect of *Fagonia arabica* on thrombin induced release of t-PA and complex of PAI-1 tPA in cultured HUVE cells

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ABSTRACT

Fagonia arabica (*FA*) possesses a thrombolytic property which has been earlier reported in our laboratory. Current study was undertaken to investigate the effect of aqueous extract of *FA* on thrombin-induced tissue plasminogen activator (t-PA) and plasminogen activator inhibitor-1 (PAI-1) release from cultured human umbilical vein endothelial cell line (HUVE) for studying its clot lytic activity. For this, establishment of cell line model has been done by isolating the cells from human umbilical cord. Cell toxicity was evaluated using XTT assay. Estimation of t-PA and PAI-1 t-PA complex were done using ELISA technique. Thrombin treatment induces the t-PA and PAI-1 release from HUVE cell line, and *FA* treatment was found to antagonize the thrombin induced t-PA and PAI-1 release. Our preliminary results suggest that *FA* may be used as an alternative to thrombolytic drug. However, study demands further experiments using animal model of thrombosis to establish the role of *FA* as a novel thrombolytic drug.
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1. Introduction

Hemostasis is the finely tuned balance between two systems i.e., coagulant and fibrinolytic system. Failure of hemostasis results in the formation of blood clot in the circulatory system.[1] According to Virchow's triad, the three broad categories of factors that contribute to thrombosis are — hypercoagulability, hemodynamic changes (stasis, turbulence) and endothelial injury/dysfunction.[2]

Thrombolytic drugs such as tissue plasminogen activator (t-PA), alteplase, recombinant tissue plasminogen activator (rc-tPA) etc. are used all over the world for the treatment of thrombotic diseases but are associated with high therapeutic cost.[3–7] On the contrary streptokinase and urokinase are cost effective,[8,9] but suffer from limitations of serious bleeding complications along with reocclusion and reinfarction in some cases.[10]

The plant kingdom represents a vast reservoir of important medicinal properties (phytochemicals) which are useful for curing various diseases.[11–13] In recent years, Indian research is focusing on evaluation of specific traditional ayurvedic medicine for treatment of disorders for which it has been prescribed for a long time in order to find its active component and also to understand the mechanism of action.[2, 6–7, 14–15] Therefore, use of herbal drugs with antiplatelet, antithrombotic and thrombolytic properties can be used as a cost effective alternative for the treatment of thrombotic diseases.[16–22]

Earlier it has been reported that herbs such as *FA* have thrombolytic properties and may be used as substitutes for available thrombolytic drugs.[7,14] However, mechanism of action by which *FA* induces the clot lysis is required to be evaluated.

Endothelial cell line has been widely used to study various biomarkers associated with thrombotic diseases. Endothelial cells play an important role in coagulant as well as fibrinolytic system by secreting various factors such as cytokines, vasoregulatory substances, adhesion molecules, growth factors, factors related to coagulant and fibrinolytic factors etc.[23]

In the regulation of hemostasis, thrombin is a central enzyme having essential role in coagulant as well as fibrinolytic system. Thrombin is directly responsible for releasing tPA from endothelial cell line which favors fibrinolysis by converting plasminogen to plasmin. Thrombin also releases PAI-1 which inhibits the activity of tPA and favors coagulation.[23] Therefore, keeping the above facts in mind, we have planned to investigate the effect of aqueous extract of *FA* on thrombin-induced t-PA and PAI-1 tPA complex release in cultured HUVE cell line to assay the clot lytic activity.

2. Materials & methods

2.1. Materials

The L-15 Medium (Leibovitz's), Medium 199, CollagenaseType I, Gelatin solution (2%), Antibiotic-Antimycotic solution (100X), HiEndo ^{XL} Endothelial Cell Expansion medium (Reduced serum), Heparin sodium salt, Trypan Blue, Trypsin solution (0.25%) were procured from Himedia. Fetal Bovine Serum (FBS), Poly-L-Lysine, Thrombin from Bovine Plasma and 2, 3 bis (2-methoxy- 4 nitro 5-sulfophenyl) 2H- tetrazolium- 5 carboxanilide inner salt (XTT) were purchased from Sigma (St. Louis, MO,USA). Other chemicals are of analytic grade. Cell culture plastic wares were purchased from Axiva Sichem Pvt. Ltd. (New Delhi, India).

2.2. Plant material

The plant *FA* (common name: Dhamasa, belongs to the family: *Zygophyllaceae*) was purchased and identified by Dr A. M. Mujumdar, Agharkar Research Institute Pune, Maharashtra, and the authentication number is Auth07-89.

2.3. Herbal preparation

Dried Herbs, *FA* was purchased from local market and was verified with the help of a Botanist and an Ayurvedic Physician. Water extract was prepared using Soxhlet extractor. The extract was prepared only once and used in all experimental set up.100 mg extract was suspended in 10 mL distilled water and the suspension was shaken vigorously on a vortex mixer. The suspension was kept overnight and decanted to remove the soluble supernatant, which was filtered through a 0.2- micron syringe filter. This filtrate obtained was used as a stock solution (10 mg/ml).

2.4. Isolation and primary culturing of HUVE cell line from human umbilical cord

All methods for the experiments included in this study were approved by Ethical Committee of Central India Institute of Medical Sciences, Nagpur. Endothelial cells were obtained from human umbilical cord vein by the method of Jaffe et.al. & Ulrich-Merzenich et.al. under aseptic conditions.[24,25] Briefly, the cord was severed from the placenta soon after birth, placed in a sterile container filled with the transport medium i.e., L-15 medium (10%FBS & 10X Antibiotic-Antimycotic solution). Before isolation the cord was carefully inspected, and all areas with clamp marks were cut off. The umbilical cords were dipped in betadine solution in the Phosphate Buffer Saline (PBS) (1:5) for 2 min followed by 70% ethanol for 1 min. Blood and blood clots from the umbilical cord were removed and fresh cuts were made on both the ends. The vein (largest opening in the cord) was perfused with 5–10 mL of cold PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM $Na_2HPO_4.7H_2O$, 1.4 mM KH_2PO_4, pH 7.4) for five-six times to wash out the blood and blood clots. Two to three millilitres of collagenase solution (1 mg/mL) were injected by the syringe (2 mL) through the needle (top winged

infusion set) with the plastic needle sheath ON, into the vein and then the needle was clamped in place with artery forceps. The other end of the umbilical cord was also clamped with an artery forcep. The umbilical cord was placed in the beaker containing PBS and incubated at 37 °C.After incubation, the collagenase solution containing the endothelial cells was flushed from the cord vein by perfusion with medium 199 (containing 10%FBS). The effluent was collected in the 15 mL centrifuge tube and was centrifuged for 10 min at 1000 rpm. Endothelial cells were cultured in the HiEndo^{XL} Endothelial Expansion Medium with 10x antibiotic-antimycotic solution and seeded in the 1% gelatin or poly −L-lysine coated T-25 flask. Cultures were identified as HUVE cell line through their cobblestone/polygonal morphology under the light inverted microscope and by the presence of von Willebrand factor (vWF).

The confluent monolayer was obtained after 7–8 days. At confluence, cells were harvested by treatment with 0.05% trypsin-0.02% EDTA, the trypsin was inactivated by the addition of medium 199 containing 10% FBS and the cells were routinely passaged at a constant 1:3 split ratio. Between three − five passages of HUVE cells were used for experiment.

2.5. Characterization of HUVE cell line from human umbilical cord

Characterization of HUVE cell line was done by estimating vWF in the conditioned medium of HUVE cells in comparison with conditioned medium of Peripheral Blood Mononuclear cell line (PBMC) as a control since they do not express vWF, while HUVE cells are reported to express and secrete considerable amount of vWF.[26−29] In brief, 100 µl of conditioned medium of sample (HUVE cell line) and control (PBMC cell line) was added to the microtitre wells and the plate was incubated at 37 °C for 90 min and thereafter blocked with blocking buffer (0.5% BSA in PBS-T). Incubation was carried out at 37 °C for 45 min. The primary antibody (vWF antibody, 1:100) raised in goat was added in the microtitre wells and the plate was incubated at 37 °C for 2 h. Then, secondary antibody (Rabbit anti-goat IgG-HRP, 1:1000) was added to the wells and incubated for 1 h. 100 µl of TMB/H_2O_2 substrate was added to the wells and incubated at room temperature for about 5–8 min. The reaction was stopped with 100 µl of 1N H_2SO_4. The absorbance of color in each well was read at 450 nm. Each sample was tested in triplicate.

2.6. Analysis of cell toxicity by XTT assay

Cell toxicity was assayed using the XTT assay as described earlier.[30] For cell toxicity assay, 2×10^5 cells/well HUVE cells were incubated with different concentrations of *FA* i.e., 100 µg/ml, 500 µg/ml & 1000 µg/ml. Briefly, after 24 h of incubation, 20 µl of XTT containing phenazine metrosulphate (PNS) (0.92 mg/ml XTT solution) was added to each well. After a four −hour incubation period, the plate was read at wavelength of 450 nm on a Stat Fax 325 + microtitre plate reader (Ark Diagnostic, Mumbai, India). Results of XTT assay were expressed in terms of percentage viability considering the viability of untreated cells (control) as 100%.

2.7. In vitro thrombosis model

The *in vitro* thrombosis model was established by the method described by Zhao et. al.[31] Passages between 3–5 of HUVE cells were used for experiment. 2×10^5 cells/well HUVE cells were cultured in the 1% gelatin/poly −L- lysine coated 24-well tissue culture plate. The culture medium was discarded and next, fresh, medium with reduced serum was added. The plates were induced with 3 U/mL of thrombin with presence and absence of different concentration of *FA* (added 10 min after the application of

thrombin) and incubated at 37 °C for 24 h. After completion of incubation supernatant were collected and stored at -80 °C for further analysis.

2.8. Estimation of tPA and PAI -1 tPA complex release by the HUVE cell line using ELISA

t-PA and PAI-1 tPA complex release, in the cell culture supernatants were estimated using the commercially available kit (Molecular Innovations, Novi, MI, USA) as per the manufacturer's instructions. t-PA and PAI-1 tPA levels were expressed in ng/2×10^5 cells.

2.9. Statistical analysis

All the statistical analysis was performed using MedCalc statistical software. Data is expressed as mean ± SD. P < 0.05 were considered statistically significant. All experiments were carried out in triplicates.

3. Results

3.1. Isolation and primary culturing of HUVE cell line from human umbilical cord

Endothelial cells were isolated and cultured in the HiEndo[XL] Endothelial Expansion Medium and seeded in the 1% gelatin or poly-L-lysine coated T-25 flask. Fig. 1 shows the confluent monolayer (obtained in 7–8 days) of long, elongated, polygonal/cobblestone homogenous HUVE cell line under the inverted light microscope.

3.2. Estimation of vWF factor by ELISA

HUVE cells and PBMC cells were isolated and seeded in the 6 wells tissue culture plate. After the cell confluency reached 70%, the conditioned medium of both the cell lines (PBMC and HUVE cell line) were collected. The characterization was done using ELISA technique. As shown in the Fig. 2, it can be observed that there is 50% increased in the vWF level in the conditioned medium of HUVE cell line as compared to the control (PBMC's conditioned medium). Thus, the primary cultured cell line was characterized as HUVE cell line.
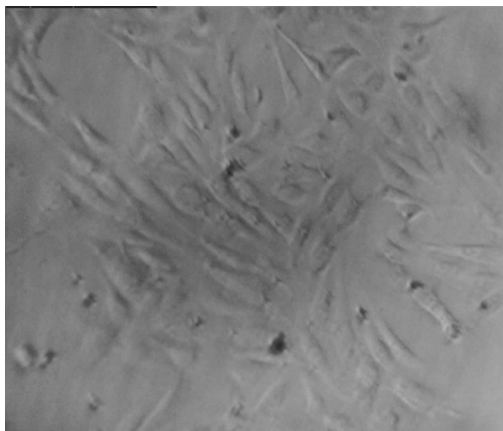

Fig. 1. Confluent monolayer of the Human Umbilical Vein Endothelial (HUVE) Cell Line. Figure shows the confluent monolayer of long, elongated, polygonal/cobblestone homogenous Human Umbilical Vein Endothelial cell line under the inverted light microscope.
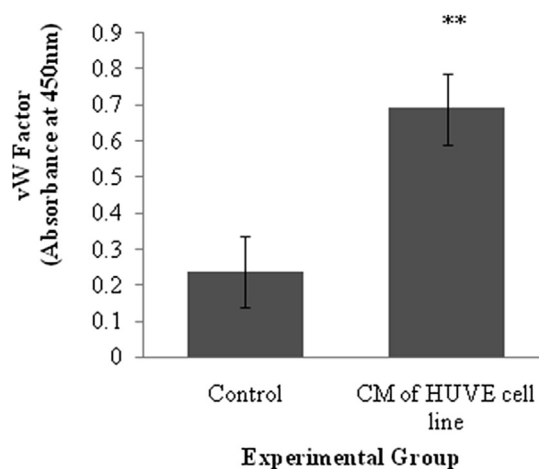

Fig. 2. Characterization of HUVE cell line. Graph represents the presence of vWF factor (marker of endothelial cell line) in the conditioned medium of the HUVE cell line. **p value<0.05, Control vs Conditioned Medium of HUVE cell line.

3.3. Results of cell toxicity assay

Results of the XTT assay (Fig. 3) shows that, FA has no toxicity effect on the endothelial cells at the concentration 100 μg/mL & 500 μg/mL, whereas 1000 μg/mL aqueous extract of FA shows 4% toxicity.

3.4. Effects of FA on t-PA release & PAI1 t-PA complex release

In the present study, we established an in vitro-model for thrombosis using thrombin. We then monitored the release of t-PA and, its inhibitor PAI-1 in the presence and absence of FA. In our preliminary study, we demonstrated that aqueous extract of FA; possessed the ability to enhance the activity of HUVE cell line to increase the release of the tPA in vitro as shown in the Fig. 4. Our result showed that thrombin induced endothelial cell line increased the activity of tPA by 1.23 fold. When thrombin (3 U/mL) induced endothelial cell line was treated with different concentration (i.e., 100 μg/mL & 500 μg/mL) of FA, the activity of HUVE cell line to produce tPA increased by 1.07 & 1.54 fold. In our experiment, to assay the free tPA concentration in the medium, we also evaluated the concentration of PAI1 tPA complex as shown in the Fig. 5. It was observed that there is 1.64 fold increase in the concentration of PAI1 tPA complex released by thrombin induced HUVE cell line. When endothelial cell line was treated with different concentration (i.e., 100 μg/mL & 500 μg/mL) of the FA extract 10 min after the addition of the thrombin (3 U/mL), there is 0.7 fold and 0.3 fold decrease in the PAI1 tPA complex.

4. Discussion

An in vitro cell line study has given the perception of understanding the cell functioning in the pathophysiologic conditions. Hence based on earlier studies, we established in vitro thrombosis model using HUVE cell isolated from human umbilical cord for evaluation of clot lytic activity of FA.[24,26,32]

In the present study, evaluation of clot lytic activity of FA with respect to t-PA and PAI-1tPA complex release from cultured HUVE cells was studied. At first, toxicity of FA was checked using XTT assay. We did not find any toxicity effect of FA on the endothelial cells. Study by Satpute et.al. (2009) has also shown that the cytotoxicity induced by ischemia is reduced to significant extent when cells are treated with FA.[33]
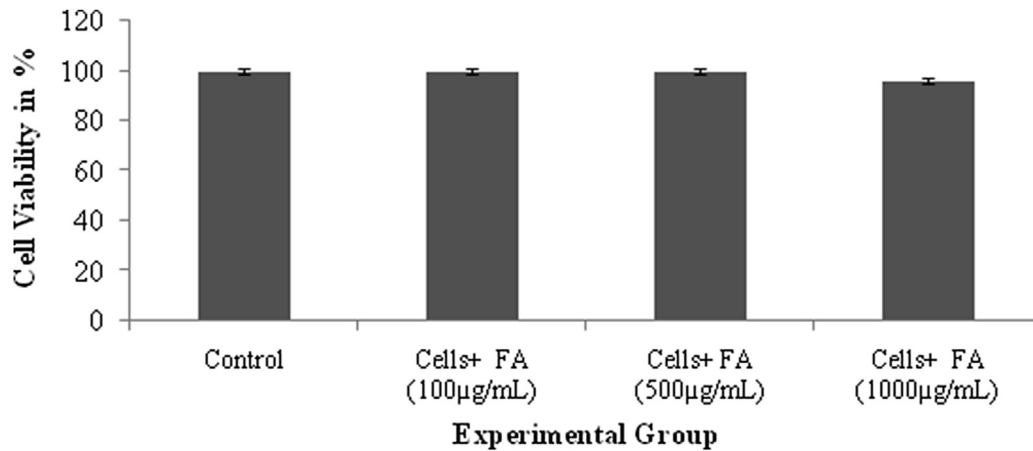
Fig. 3. Effect of *FA* on HUVE cell line. Graph represents effect of the three experimental groups of different concentration of FA on cell viability in terms of percentage.

Thrombin is a serine protease enzyme which has an important function in regulation of coagulation and fibrinolytic system. We found that thrombin induced HUVE cells secrete both tPA as well as PAI-1 which was in accordance with the previous report.[34,35] The

Fig. 4. Effect of *FA* on the concentration of total t-PA in the conditioned medium of thrombin induced HUVEs. Graph represents the mean of the two different concentrations of FA,*p < 0.05 vs. control group, #p < 0.05 vs. thrombin induced HUVE cells group.

secretion of tPA by a constitutive and a regulated pathway only occurs, when the receptors on endothelial cells gets activated as reported in earlier studies.[36,38] But in general PAI1 level is several fold higher as compared to tPA.[37] Under physiological conditions, PAI1 is primary inhibitor of tPA.[36] We also found that the level of total tPA increased as compared to PAI-1 tPA complex when the thrombin induced HUVE cell line was treated with different concentrations of *FA* in our experimental study.
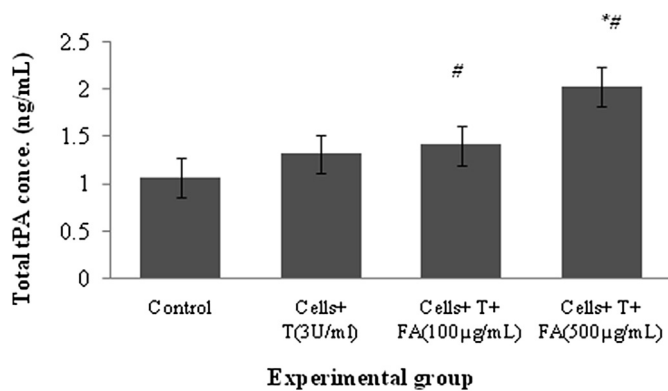
Earlier several *in vitro* studies have explained that inhibition of the PAI-1 release favors the fibrinolytic activity. Flavonoids and phenolics isolated from plant origin are potent inhibitors of the PAI1 production in cultured HUVE cell line (Kimura et. al 1997; Zhao et al. 1999).[39,31] The seeds of *Nigella sativa* (NS) L. (*Ranunculaceae*) have been shown to regulate fibrinolysis/thrombus formation by modulation of fibrinolytic potential of endothelial cell line as reported earlier.[40] Four medicinal herbs Danshen (DS, *Salvia miltiorrhiza*), Shanchi (SQ, *Panax notoginseng*), Shanzai (SZ, *Hawthorn*) and Heshouwu (HSW, *Polygonum multiflorum Thunb*) in traditional Chinese medicine have been shown to have activity that may contribute to cardiovascular protection by Ling et al. (2008).[41]

Similarly, we also found that treatment of *FA* favors the acute release of tPA and enhances the availability of the free tPA from
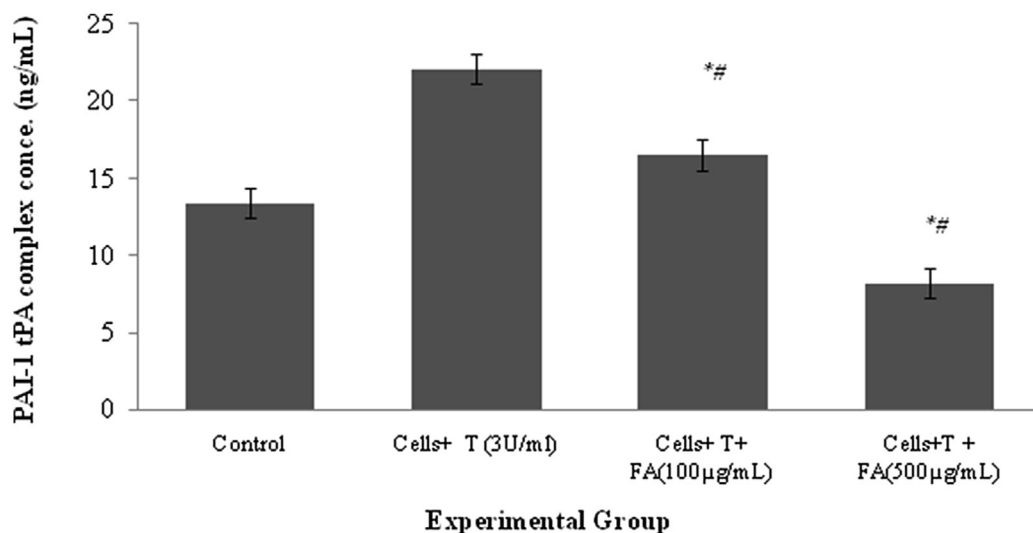

Fig. 5. Effect of *FA* on the concentration of PAI-1 t-PA complex in the conditioned medium of thrombin induced HUVEs. Graph represents the mean of the two different concentrations of FA,*p < 0.05 vs. control group, #p < 0.05 vs. thrombin induced HUVE cells group.

thrombin induced HUVE cell line culture by inhibiting the release of PAI-1.

To the best of our knowledge this is the first report describing the clot lytic mechanism of *FA* in the HUVE cell line model. However, study demands further experiments using animal model of thrombosis to establish the role of *FA* as novel thrombolytic drug.

5. Conclusions

The preliminary study suggests that *FA* favors fibrinolysis in *in-vitro* cell line model which confirms our earlier findings with *in vitro* test tube model. Thus, *FA* can be used as a novel cost effective and safe alternative to currently available thrombolytic drugs.

Conflict of interest

Authors declare no conflict of interest.

Acknowledgments

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