Summary

Thrombosis, the formation and development of a blood clot within the vascular system is a life saving process only when it occurs during a hemorrhage. But clot formation and its migration can be life threatening when it occurs either inside the blood vessels of brain (causing thrombotic strokes) or the coronary artery causing heart attacks. According to the World Health Organization (WHO) latest data sheet (May 2017), Cardio Vascular Diseases (CVDs) are the number one cause of death globally. In India, stroke has increased significantly in both urban and rural areas (Gupta, Joshi et al. 2008) and according to an estimate form The Global Burden of Disease study, CVD became the leading cause of mortality in India. CVD death rate in India is 272 per 100000 population compare to the World death rate 235 per 100000 populations. By the year 2030 every 5th person affected from CVD will be an Indian (Gupta, Mohan et al. 2016; Prabhakaran, Jeemon et al. 2016).

CVD is a class of diseases which involves heart and blood vessels- like myocardial infarction, pulmonary embolism, deep vein thrombosis and stroke. The most preferred emergency medication against thrombotic circulatory disorders are the plasminogen activator protein drugs such as Streptokinase (SK), Staphylokinase (SAK) and tissue plasminogen activator (tPA) (Lijnen and Collen 1988; Collen and Lijnen 1990; Francis and Marder 1991). They have been slowly losing their supremacy to emergency cardiac interventions such as stenting and bypass surgeries in the affluent countries because of bleeding risks associated with their use, and also the often-encountered problem of clot reformation at the same site of vascular injury due to thrombin activity and/or fresh generation of thrombin. This is particularly the case with ischemeic strokes. Indeed, the relevance of an optimized clot buster for this malady is even more acute than before; the experience with tPA has shown both the strength of thrombolytics as well as their limitations in this case. A woeful lack effective of surgical interventions, on the one hand, and the failure of the only lytic approved (tPA), after a 2-3 hour "Golden Window", are two challenges that need urgent addressal.

One of the most common early-stage medications in animals/humans such as myocardial infarction is the intravenous infusion of thrombolytic agents such as tissue plasminogen activator, Urokinase and streptokinase (Lijnen and Collen 1988). Among these, SK is a commonly used thrombolytic drug in developing countries because of its low cost and potent thrombolytic agent. It is a bacterial protein which helps convert human plasminogen into

plasmin, which dissolves the clot (Bajaj and Castellino 1977; Banerjee, Chisti et al. 2004) but acts as an indirect activator of plasminogen (De Renzo, Boggiano et al. 1967).

According to the World Health Organization (WHO) report, myocardial infarction and stroke are now more prominent diseases in developing and poor countries. On the contrary, both myocardial infarction and stroke are better managed in developed countries due to the availability of sophisticated Cath labs and quick SOS responses of professional medical facilities that save the several lives. Currently available thrombolytic drugs only can be given under the medically well-equipped hi-sophisticated life saving settings due to thrombolytic administration rapid generation of plasmin causing several unwanted episodes at the cellular and molecular level which starts squeal of uninvited events such as hypercoagulability of blood, hemorrhage and reformation of clots after thrombolytic therapy. In past two decades, these clinically challenging problems have attracted continuous attention and serious efforts towards this direction have been taken albeit with limited success. Although advancements towards the development of novel drug regimens such as the combined use of lytics and anti platelet therapy has been seen, and several investigative models and their evaluation through sophisticated instruments have given the mechanistic insight towards the understanding of the pathophysiology of the disease, there is an acute need for "breakthroughs" in the design of multi-functional thrombolytics to combat the challenges in the clinic. Among thrombolytics only tPA and SK, and more recently tenecteplase are approved for the medical intervention in MI. Beside these, some fusion constructs with SAK (Kowalski, Brown et al. 2009; Wang, Zhang et al. 2009) and vampire plasminogen activator (Liberatore, Samson et al. 2003) have been made and reported in the literature with their beneficial properties but these never came to successful human trials. A key requirement toward the successful thrombolytic is an affordable cost. Tissue plasminogen activator which is produced by animal cell culture, costs around ~30000 rupees in Indian market whereas streptokinase or recombinant streptokinase is around ~1500 rupees. That's why not only in India but in other poor countries streptokinase becomes the preferred choice of the drug. However SK is not allowed for strokes due to its lack of fibrin clot specificity.

Successful thrombolytic therapy helps in maintaining normal blood flow and improves the survival in a significant number of patients (Verstraete 1990), but early re-occlusion or re-thrombosis, often at the same site, has continued to limit the successful application of thrombolytic drugs especially in stroke. Several studies demonstrate that early re-occlusion

occurs in upto 10-15% of patients after thrombolytic therapy (Ohman, Califf et al. 1990) as, during lysis, the transiently released thrombin initiates a self generation loop to amplify clot growth.

Apart from promoting procoagulant activities in blood, thrombin also plays a central role in the management of indirect thrombin's regulators via activation of the well known Protein C activating pathway. In order to control the level of indirect regulators, free thrombin makes a 1:1 high affinity, non-covalent complex with thrombomodulin, a cell surface protein (Kurosawa, Galvin et al. 1987), and once this thrombin-thrombomodulin complex is formed it redirects the thrombin's substrate specificity from a procoagulant mode to an anti-coagulant mode, whereby it activates the Protein C anticoagulant pathway. Thus, even though thrombin alone can activate Protein C, but once it complexes with thrombomodulin it accelerates Protein C activation by nearly a 1000-fold (Esmon and Owen 1981; Owen and Esmon 1981).

Some time ago, a patent namely "Protein fusion constructs possessing thrombolytic and anticoagulation properties" from Dr. Sahni's group (US Patent 9150844) described the preliminary expression and purification properties of antithrombin fusion constructs that showed accelerated plasminogen activation in presence of trace amount of plasmin and directed significant thrombin inhibition properties along with anticoagulant Protein C activation capability. These phenomena are unexpected from point of view solely of the native streptokinase activity, but if one could integrate these properties onto the "robust" SK scaffold with its high turnover plasminogen-to-plasmin conversion, these combined properties (plasmin generation, clot specificity, direct thrombin inhibition, inhibition of thrombin's procoagulant properties) could be immensely useful for the treatment of myocardial infarction as well as stroke. In order to further validate and evaluate the properties if SK based these fusion constructs new experiments have been designed and comparison with streptokinase has been carried out in reasonable detail.

In our simulated, purified fibrinogen clot lysis system when plasminogen activation was seen, some delay in clot lysis was observed due to plasmin-dependence following pathway II for activation. The *in vivo* milieu, on the other hand, with fibrinogen present in a full protein complement plasma system, has ~10 fold higher plasminogen concentration at and around the clot. Therefore, real *in vivo* conditions cannot be fully simulated in a 'mere' microtiter plate with a plasminogen activation-clot lysis system reconstituted from purified components. Another reason of not using plasma instead of fibrinogen in the mircrotiter plate is due to the

presence of porphyrins in plasma imparting slightly yellowish in color with its attendant absorbance at 405 nm and its persistence during clot preparation and lysis, such that it strongly interferes with the assay. Therefore, we decided to design appropriate *in vivo* conditions where clot lysis can be measured without the complication from the presence of plasma *per se*. In literature, iodine-^{125} (I-125) radiolabeled fibrinogen was traditionally incorporated into the fibrin clot after thrombin mediated coagulation, and lysis by the lytic agent was detected by the counts emitted by I-125 in the soluble phase (Moroz and Gilmore 1975). This method is quite cumbersome and radioactivity disposal is a tedious job, therefore FITC labeled fibrinogen was used to be incorporated in the clots. The excitation and emission of fluorescein dye during the lysis was measured by spectrofluorometer. Surprisingly, NEGFSK showed somewhat faster clot lysis compared to SK. One plausible reason is NEGFSK contains thrombin binding domain so it binds to the thrombin present in clot resulting in faster clot lysis. Second possible reason is plasminogen and tissue plasminogen activator get accumulated at the clot and they form trace amount of plasmin at the clot surface; therefore, NEGFSK is activating only at the near vicinity of the clot. Once SK binds to the plasmin it will degrade to different degrees (Aneja, Datt et al. 2009; Yadav, Aneja et al. 2011) whereas NEGFSK is not activating the free plasminogen; hence it may survive for a longer time and activate plasminogen when it comes in contact with clot surface. These indicate that NEGFSK may not be directly involved in plasminogen activation but *in vivo* like situations it assumes functions of a potent clot dissolver.

Like free plasmin, free thrombin never exists beyond a very short duration in blood/plasma as it is rapidly neutralized by the anti-thrombin (AT) and heparin complex (Naski and Shafer 1993). In a reconstituted *in vitro* plasma system, it is difficult to prove whether these NEGFSK construct will bind to the clot-bound thrombin. Therefore, we again resorted to the purified system where fusion construct was incubated with clot formed by purified fibrinogen, washed and suspended in buffer containing plasminogen, wherein the excess amount of construct will be washed out and plasminogen was added to see the presence of thrombin-clot-bound fusion constructs mediated clot lysis. Results obtained clearly indicate that NEGFSK is showing faster clot lysis, this indicates that the fusion construct is capable of binding with clot-bound thrombin.

Another major problem, associated with the available FDA-approved-thrombolytics (tPA and SK), is that during therapy rapid decline in fibrinogen has been observed (Vandelli, Marietta

et al. 2015). Keeping this thing in mind fibrinogen estimation method was used and found that only NEGFSK is capable of providing fibrinogen protection over a broad therapeutic range with time, as compared to SK. It is clear now that immediate plasminogen activation property of thrombolytic is causing the rapid decline in plasma whereas plasmin dependent fusion construct was not showing any rapid decline of fibrinogen. Thus, the most coveted property of fusion construct is maintaining good fibrinogen level fibrinogen by stopping rapid plasminogen activation. This property may help in the prevention of hemorrhage and allow administering it in a single bolus due to its remarkable clot selectivity and safety profile which is not present in SK.

We know that reformation of clot is initiated even after successful clot lysis process. Plasmin is a non-specific protease so it is possible that it can degrade/cleave the EGF4,5,6 domains and it may not prevent the re-occlusion actually in a proteolytically active milieu. Keeping this problem in mind a new assay was designed where clot formation and clot lysis were carried out in purified fibrinogen buffer, and clot lysis step was arrested at 50 % clot lysis that allows the survival of significant remnants of clot or active partial clot surface (simulating *in vivo* conditions) that contain entrapped thrombin which can potentially initiate the clot reformation process. The entire assay was carried out at in a small microtiter plate hence close contact of fusion constructs and plasmin is more, therefore, the probabilities of possible chopping off the fusion construct was also more. Here, lytic condition was stopped by rapid washing and remaining plasmin activity is quenched by the addition of α_2-antiplasmin. Even in such conditions, the fusion construct was showing 22-25% less re-occlusion as compared to streptokinase thus strongly reinforcing the improved property in the protein engineered construct.

Clot lysis potential of NEGFSK is equal to the SK in plasma based clot lysis system. To further investigate its clot lysis potential in blood, mouse blood clots were prepared and thrombolytic assay was performed. In this monitoring of lysis of halo shaped blood clots was observed via change in absorbance due to release of blood cells in well plate. The result showed that NEGFSK is not capable to activate mouse plasminogen same as SK but when human plasminogen was added to the system it starts clot lysis after some lag phase. In SK the lysis starts early compared to NEGFSK which also indicate its plasmin dependency to activate plasminogen.

Earlier granted lab patent, it was demonstrated that the crude supernatant containing NEGFSK fusion protein has ability to activate Protein C. Now we wanted to check whether purified NEGFSK showing Protein C activation when incubated with thrombin? The *in vitro* assay with free thrombin showing Protein C activation indicated that the EGF domains of NEGFSK is capable to bind with free thrombin and further activating Protein C. But clot bound thrombin is main culprit that initiate reformation of clot at the same site (Maki et al., 1998; Meissner et al., 1995; Prins and Hirsh, 1991; Tyczynski et al., 2014), therefore, an assay was devised to check that whether clot-bound-thrombin is capable to bind with NEGFSK and activating Protein C. In order to evaluate human EGF 4,5,6 domains, in chimeric construct NEGFSK, mediated protein C activation in clot bound thrombin; mouse blood clot was prepared by adding human thrombin. In this experiment human plasma/blood cannot used because SK component which specifically activated human plasminogen may also cleave the Protein C specific chromogenic peptide whereas human specific-streptokinase unable to activate mouse plasminogen, hence, this system specifically evaluate the Protein C activation with human thrombin. In results mouse blood clot made by human thrombin was not lysed during incubation with SK and NEGFSK. In Protein C activation assay NEGFSK successfully activates Protein C into activated Protein C whereas streptokinase alone fails to do. This proved that the EGF domain is capable of activating Protein C by binding with clot-bound thrombin and this property is also beneficial for preventing reocclusion of clots due to clot-bound thrombin mediated Protein C activation.

Another important parameter that widely studied that activation of MMP -9 during the tPA intervention while treating ischemic stroke. Various well-studied literature suggested the role of MMP-9 get up regulation in stroke and level of MMP-9 increased while tPA intervention. It has been shown and validated that tPA causes hemorrhage and blood brain barrier destruction by up regulating the MMP-9 expression even administered within therapeutic window (1995; Adibhatla and Hatcher, 2008; Aoki et al., 2002; Cronin, 2010; Hacke et al., 2004; Lyden et al., 1989; Tsuji et al., 2005; Wang et al., 2003). This causes damage to the brain and sometimes causing a life threatening condition. In order to check whether SK and NEGFSK will up regulate or down regulate the expression of MMP-9 in stroke we performed transient Middle Cerebral Artery Occlusion (MCAO) in mouse that mimic stroke like Oxygen Glucose deprived (OGD) conditions. Brain samples were used to check the level of expression of MMP-9 protein by western blotting method. Results suggested that tPA was up

regulating MMP -9 expression 2.5 fold compared to positive control where only PBS was injected in place of drug. In case of SK, MMP-9 expression was similar to PBS control (positive control) that indicates SK is not up regulating the MMP-9 expression level. Interestingly, NEGFSK intervention as a drug showed the half MMP-9 level of expression as compare to SK and PBS control. This might be due to the EGF 4,5,6 domain but here detailed mechanism of MMP-9 expression was not studied. But this clearly indicates that NEGFSK is definitely far better drug as compare to tPA in case of stroke and other blood obstruction type maladies.

In newly designed chimeric construct it was found that this molecule activate plasminogen in presence of plasmin and trace amount of plasmin is always present at the surface of clot under the physiological condition so it having a novel clot-specificity mechanism that is not reported in literature. Due to specific mechanism it show better fibrinogen protection as compare to SK, hence, it can prevent hemorrhage because a good amount of circulating fibrinogen will seal the leaked blood vessel. Further indirect studies suggest this protein is capable to bind with clot bound thrombin and clot lysis potential is similar like SK. Moreover, thrombin inhibition under high proteolytic shear condition make it versatile molecule that can prevent reocclusion. Apart from study several *in vitro* studies it is very difficult to study plasminogen activation and thrombin mediated Protein C study in *ex vivo* system. Therefore, mouse blood clot formed with the help of human thrombin was used to evaluate the protein C activation at the clot surface and results clearly indicate protein C activation. Apart from that one study that indicated that MMP-9 increased level is really harmful and generally it's level increase during the tissue plasminogen activator intervention, hence, oxygen glucose deprived model (suture base) mimic stroke like condition indicate that it does not up regulate MMP-9 level whereas tPA drastically increase the level of MMP-9. From this part of research it can be concluded that this chimeric molecule is not only retain its both the properties of fusion but far better than the existing thrombolytic agents. These properties are definitely useful for the stroke and other CVDs such as myocardial infarction and deep-vein thrombosis and dual function- clot lysis and thrombin binding and mediate Protein C activation can treat early re-occlusion problem efficiently. Although this study is just a small step towards the making of a safer and better version of thrombolytic protein which needs further investigations in appropriate animals and human trials but the preliminary results indicate that NEGFSK has successfully exhibited unique properties that

has the potential to revolutionize the thrombolytic therapy area in ischemic stroke where there is currently a yawning gap in therapy. Due to high safety (clot specificity) profile, it might be given in a single bolus injection for patients where sophisticate medical facilities are not available. The next big challenge is process development and the protein's toxicological study which will help to establish its potential more strongly.