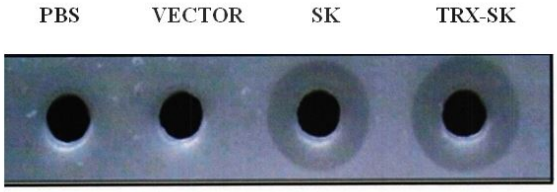
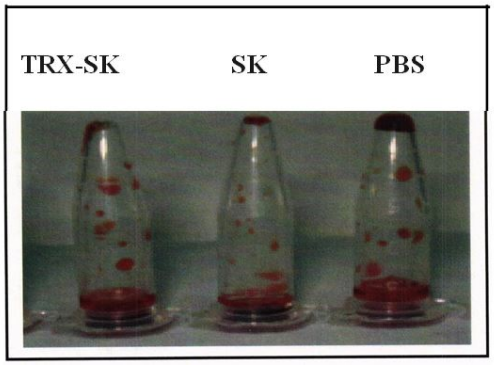
Bioassay for Streptokinase-Caseinolytic Assay.



Clot lysis Assay



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Radha	37	F	1.31	1.16
Ramalingam	42	M	1.3	1.15
Ganasundari	41	F	1.31	1.15
Murugammal	40	F	1.31	1.15
Murugammal	39	F	1.3	1.15
Vijaya	42	F	1.3	1.15
Rajalakshmi	40	F	1.3	1.14
Bharathi	38	M	1.3	1.15
Subramani	41	M	1.31	1.15
Fathima	40	F	1.31	1.14
Manoharan	60	M	1.32	1.15
Dhanam	56	F	1.3	1.15
Saradha	42	F	1.3	1.14
Kandhasamy	40	M	1.3	1.14
Srinivasan	38	M	1.3	1.15
Krishnan	63	M	1.31	1.15
Prema	40	F	1.32	1.15

Statistical analysis

The age of the subjects was ranging from 37-63 years with mea ± SE as 42.90 ± 1.6 years. 85% were less than 50 years and 15% greater than 50 years.

The Clot lysis activity of Streptokinase for age less than 50 years was observed to be 1.3± 0.001 (mean± SE) and for age above 50 years 1.31± 0.005(mean± SE) yielding a statistically insignificant t value of 1.39(p=0. l 79).

The Clot lysis activity of Thioredoxin-Streptokinase for age less than 50 years was observed to be 1.14± 0.00l(mean± SE) and for age above 50 years 1.15± 0.000 (mean± SE) yielding a statistically insignificant t value of 0.709(p=0.488).

Table 3: Statistical analysis of clot lysis time with age.

PROTEIN	AGE (years)	CLOTLYSIS TIME (Hours)	T value	P value
STREPTOKINASE	<50	1.30 ± 0.001 I	1.39	0.179
	>50	1.31 ± 0.005		
THIOREDOXIN-STREPTOKINASE	<50	1.14 ± 0.001	0.709	0.488
	>50	1.15 ± 0.000		

Table 4: Statistical analysis of the clot lysis time with sex.

PROTEIN	Sex		T value	P value
	Male	Female		
STREPTOKJNASE	1.30 ± 0.002	1.30 ± 0.001	0	1
THIOREDOX.IN-STREPTOKINASE	1.14 ± 0.0012	1.14 ± 0.0017	0.513	0.614

DISCUSSION

Streptokinase is a monomeric multi domain protein of 47 kDa produced by several hemolytic strains of streptococci. Streptokinase (SK) is a potent plasminogen activator with the widespread clinical use as a thrombolytic agent. Handling this status with a thrombolytic alone cannot provide a complete solution as the part of ischemia mediated free radical injury is left unattended. Thioredoxin, a REDOX protein forms the free radical scavenging system in the human body. It has the ability to reduce disulfide bonds that do not respond to

other cellular reductants52• Furthermore, it has been demonstrated that thioredoxin reductively inactivates phospholipaseA2 which is one of the contributing factor in inflammation and is also known to promote angiogenesis. In research, thioredoxin can be used in the over expression of genes encoding economically valuable proteins and peptides. The thioredoxin_streptokinase fusion construct thus designed so as to develop a synergetic protein of higher potency in the management of myocardial infarction.

In order to achieve maximum expression, a highly efficient system like T7 was employed in pRSETA expression vector. The T7 expression vectors are designed for high level expression, simple cloning, specific detection and efficient purification of expressed proteins. These vectors contain strong T7 promoter which provides high level expression of the target protein53 and also the presence of polyhistidine allows the target protein to be expressed as fusion proteins which facilitates the single step purification using chromatography techniques[5].

High level expression is achieve- d in T7 expression systems because the T7 RNAP is more processive than E.coli RNAP and is dedicated to the transcription of gene of interest. The T7 RNAP gene is under the control of the lacUV5 promoter which can be induced by IPTG. Therefore the expression vectors contain one of the T7 promoters to which the recombinant gene will be fused. In this case TRX-SK gene was fused to an inducibile promoter allowing its transcription and translation during expression phase. However, disadvantages still exist, such as formation of inclusion bodies in the cell cytoplasm. The pRSETA vector replicate from PUC1 origin of replication which maintains a high copy number per cell. So the host that harbours pRSETA construct has high gene dosage of the target protein. The literature reports that using a high copy number plasmid for the expression of the target protein has no advantage over the plasmids that replicate from another origin of replication(such as pBR322). However, the plasmid copy number preferably replicates in a relaxed fashion. These multi-copy numbers are stably replicated and maintained under selective conditions and plasmid fre-e daughter cells are rare.

In this study, we have integrated thioredoxin with streptokinase and the expression of TRX-SK in E.coli was carried out at different temperatures (25°C, 30°C, 35°C and 37°C), different post induction time(3 hrs and 6 hrs) and optica: .. ensities (0.6 O.D and 1.0 O.D) in BL21 (DE3) and GJl 158 expression hosts. Hyper expression was achieved in GJl 158 at around 0.6 O.D. at a post induction temperature of around 30°C.

Inclusion body formation remains a significant barrier to gene expression in the cytosol, however inclusion bodies do offer several advantages. However, these are small consolation considering the arduous task of refolding the aggregated protein (Rudolph and Lilie 1996), the uncertainty of whether refolded protein retain its biological activity and the reduction in the yield of the refolded and purified protein to date, the precise physico-chemical parameters that contribute to the formation of inclusion bodies remain unclear 55 56 57 Inclusion bodies formation can be minimized by several methods, such as co expression of molecular chaperons, choosing appropriate host, decreased cultivation temperature, increasing aeration, medium composition, low inducer concentration, and induction at low cell densities58• In E. coli GJl158, the pro U promoter governs the expression of T7 RNA polymerase which is induced by addition of NaCl. Medium used for the growth of the strain is devoid of NaCl hence hypoosmotic, on induction by the addition of NaCl b mes hyperosmotic. Under such conditions of hyperosmolarity, osmo responsive proteins that are involved in praline transport are induced and the intracellular levels of osmoprotectants such as praline, glycinebetaine have been known to increase in E. coli 59. Such osmoprotectants have been known to improve protein folding. Improperly folded proteins are toxic to the host and hence there is degradation of the proteins (Kagawa and Cao 2001, Savvas Makrides 1993). Improper folding may be related to improper formation of disulphide bonds. The E. coli GJl 158 has been shown to improve folding and activity of recombinant proteins60 case of E. coli GJl 158, the environment of osmoprotectants like praline, glycinebetaine may help in expression by conferring better folding and stability.

Expression of the soluble protein optimized and large amount of the soluble protein around more than 50% of the soluble protein was obtained at around 30°C (3 hrs induced samples) at 0.6 O.D. in GJl 158. The soluble fraction was subjected to anion exchange chromatography to yield a single band in SDS PAGE analysis and was further confirmed by western blot. The dithiol-disulfide oxidoreductase activity of thioredoxin for the regulation of enzyme activities (Holmgren 1989) can be assayed by the catalysis of the reduction of insulin disulfides with dithiot teitol. In this reaction, the rate of insulin reduction observed spectrophotometrically at 650 nm at 25°C as turbidity formation from the precipitation of the free insulin chain.[26] The caseinolytic and the clot lysing activity of the recombinant protein is compared with that . of the control Streptokinase. The positive clot lysis assay and caseinolytic assay confirms the retention and enhanced biological activity of the constituent Thioredoxin and Streptokinase of the recombinant fusion protein. Thus it was attempted to generate a recombinant protein with both antioxidant and thrombolytic property taking care of both the thrombotic status and the ischemia mediated free radical injury and promoting angiogeogenesis in the thrombo-embolic disorders like Myocardial Infarction, Cerebrovascular accidents etc.

CONCLUSION

Cloning the thrombolytic streptokinase along with a redox protein like thioredoxin gives the benefit of free radical scavenging effect on the ischemia mediated free radical injury of the vascular endothelium and surrounding cells. This can find potential usage in thrombo-embolic disorders like Myocardial Infarction,

Cerebro Vascular Accidents etc... where the ongoing oxidative damage and reperfusion injuries can be handled along with thrombolysis.. The following were achieved by c rying out this work: Amplification of human Thioredoxin gene from lung cancer cell line A549. Cloning of human Streptokinase in pRSET A at Barn HI and Eco RI site and Fusion of human thioredoxin in the recombinant clone pRSET Al SK at Ndel and Barn HI site.

Expression of the hybrid streptokinase in BL21 (DE3) and GJ1158 and Optimization of expression at various inducer concentrations, different temperature, medium and hosts. Solubility studies & Purification studies. Bioassay for the recombinant protein in the form of Insulin reduction assay, Caseinolytic assay and Clot lysis assay were performed. The results showed that the recombinant protein is more effective than plain Streptokinase.

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DECLARATION OF CONFLICT OF INTEREST

The authors declare no conflict of interest.

FUNDING

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ETHICAL APPROVAL

The study was approved by the Institutional Ethics Committee.

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