Chapter V

Invitro culture of neutrophils to study the effects of triggering factors on elastase release from neutrophils
Introduction

Non-infectious diseases included in this study are diabetes mellitus, preeclampsia and stroke. A common observation noted among them were increased levels of NE and a positive correlation of NE with the causative and/or risk parameters of all disease conditions. It is known that hyperglycemia leads to release of NE through inflammatory mediators, leading to complications of diabetes (135). Further, it is established that increased glucose levels could release elastase in Invitro culture system (54, 210). On the other hand, hyperhomocysteinemia is considered either as a co-morbid condition or a modifiable, independent risk factor in the development of cardiovascular diseases such as stroke, coronary artery disease, preeclampsia and diabetes (55, 56, 57).

Homocysteine (Hcys) is a thiol- group containing amino acid intermediate in methionine synthesis and is utilized in the body for transmethylation reaction. Hcys above the physiological levels exerts diverse deleterious effects on vascular and immune cells through synthesis of ROS leading to endothelial injury, platelet activation, smooth muscle cell proliferation, oxidation of LDL and induction of endothelial-monocyte interactions (211, 212). Invitro study by Bryushkova et al. demonstrated expression of N-methyl-D-aspartate (NMDA) receptors by neutrophils on their membranes and subsequent degranulation upon incubation with Hcys (213). Alvarez-Maqueda M et al also showed Hcys increased intracellular H$_2$O$_2$ via NADPH oxidase leading to activation and phosphorylation of mitogen-activated protein kinases (MAPKs) resulting in chemotaxis and migration of human peripheral neutrophils (214). In a comprehensive study conducted by Joshi et al it was shown for the first time that Hcys acts as a potential inducer of NET formation and associated release of elastase (55). As briefed above, Hcys has been implicated as a stimulating factor for neutrophils leading to pathophysiological
changes in the body. These reports prompted for designing a simple experimental setup to look for the effect of Hcys on neutrophils and elastase activity by using varying physiological and pathological concentrations of Hcys. To ensure that the system designed is dependable, a validation was carried out by using varying concentrations of glucose as a reference molecule.

**Materials and methods**

**Chemicals and Reagents**

All reagents used were of analytical grade unless otherwise specified. 2% Fetal Bovine Serum (2% FBS) (Cat. No. 10270106) and Roswell Park Memorial Institute -1640 media (RPMI-1640) (Cat. No. 31800022) were purchased from Invitrogen. Ficoll-paque Plus was purchased from GE Healthcare (Cat.No. 17-1440-02), 20% dextran was from Himedia (Cat.No. RM 4187) and 96-well tissue culture plates from Genetix. DL-Homocysteine was procured from Sigma, USA (Cat.No. 44925).

**Isolation and Invitro culture of neutrophils**

Ficoll- dextran density gradient method was employed for the isolation of human PMNs as described by Joshi et al (210). PMNs were isolated from blood of healthy volunteers with informed consent. Briefly, 5ml of blood was collected in sodium heparinized tubes and was transferred to a 15ml falcon tube and diluted it with 2ml of phosphate buffer saline (PBS). Diluted blood was gently layered on top of 4ml of Ficoll-paque Plus gradient solution in a fresh falcon tube and centrifuged the tube at 2000rpm for 20 minutes at room temperature. Top two layers were discarded carefully without disturbing the bottom layer. Cell pellets were suspended in 9ml of Hank’s balanced salt solution (HBSS) and 0.5ml of 20% dextran followed by gentle mixing. After incubation for 1 hour within laminar air flow hood, the supernatant was carefully transferred into a new falcon
tube and centrifuged at 1800rpm for 10 minutes at room temperature. Supernatant was
discarded and 2ml of RBC lysis buffer (1X) was added to the pellet. Pellet was mixed
carefully and incubated for 3-4 minutes at room temperature. This was followed by
addition of 9 ml of HBSS buffer and centrifugation at 1800 rpm for 10 minutes at room
temperature. Supernatant was discarded and the white pellet obtained consisted of PMNs.
Pellets were then suspended in appropriate volume of HBSS to adjust the concentration of
neutrophils to 2.5x10⁶ cells per ml. An aliquot was used for counting neutrophils by
Trypan- blue dye exclusion method using hemocytometer and for cell purity assessment
by Giemsa staining.

Cell suspension was centrifuged at 1800 rpm for 10 minute at room temperature,
supernatant was discarded and the pellet was resuspended in 1 ml of RPMI with 2% FBS.
Cells (2.5x10⁶) were then seeded on 96-well tissue culture plates and were incubated
without or with increasing concentrations of glucose (5.5mM, 11mM, 22M/L) and
homocysteine (5, 11, 25, 50, 200µM/L) in a final volume of 100µl per well at 37°C in
CO₂ incubator for 2hours at an interval of minutes. Three independent experiments were
carried out for each concentrations and incubation time. Supernatants were collected after
each incubation period and elastase activity quantified immediately using STANA as
substrate.

Results

Influence of glucose on elastase release: To assess the impact of high glucose
conditions on the release of elastase from neutrophils, isolated neutrophils from healthy
individuals were cultured in the presence and absence of varying concentrations of
glucose. The result obtained is represented in Fig 16.
Influence of Glucose on elastase release

The results showed that, the increase in glucose concentrations had direct effect on elastase release which is reflected in terms of increased elastase activity. On exposure to 5.5mM glucose, maximum elastase activity of 0.588±0.02 U/ml/min was recorded after 90 minutes of incubation, while 11mM glucose yielded maximum activity of 0.855±0.06 U/ml/min measured after 60 minutes of incubation followed by a decreased elastase activity (0.658±0.00 U/ml/min) after 90 minutes of incubation. On incubation with 22mM glucose concentration maximum activity was recorded at both 60 (0.946±0.04 U/ml/min) and 90 (0.948±0.04 U/ml/min) minutes of incubation. At all the provided conditions, up to 90 minutes of incubation there was an increase in elastase activity but after 120 minutes the activity decreased considerably (0.448±0.04 with 5.5mM; 0.42±0.00 with 11mM and 0.57±0.01 with 22mM of glucose).

Influence of Hcys on elastase release: Freshly isolated peripheral neutrophils were exposed to varying concentrations of Hcys and the culture supernatants were assayed for elastase activity at different time intervals. The results obtained are represented in Fig.17.
Fig 17: Influence of Homocysteine on elastase release

As indicated, elastase activity gradually increased with time at every concentration and maximum activity was recorded at 200µM/L Hcys concentration with 120 minutes of incubation. Further increase in Hcys concentration led to inhibition of elastase release. The elastase activity recorded are as follows: for 5µM/L Hcys concentration - 0.306±0.01; 0.373±0.07; 0.568±0.11 and 0.618±0.01U/ml/min; for 11µM/L- 0.347±0.07; 0.428±0.02; 0.722±0.02 and 0.726±0.05U/ml/min; for 25µM/L- 0.356±0.05; 0.394±0.09; 0.572±0.48 and 0.788±0.09U/ml/min; for 50µM/L- 0.37±0.23; 0.491±0.10; 0.681±0.23 and 0.822±0.04U/ml/min and for 200µM/L- 0.333±0.09; 0.446±0.02; 0.578±0.003 and 0.838±0.01U/ml/min after 30, 60, 90 and 120 minutes of incubation respectively.

Discussion

Deregulation of metabolic and immune response pathways in T2DM has been studied well. Neutrophils play an important role in innate immune response (7). Clinical studies and data from experimental models suggest hyperglycemia induced neutrophil chemotaxis, phagocytosis and bactericidal properties (215). Lowering of blood glucose
levels using anti-diabetic drugs has been shown to improve and re-sensitize neutrophil activity (216). Joshi et al demonstrated that a concentration dependent increase in glucose levels (15mM and 20mM) significantly increased NETs formation (210). Menegazzo L also observed increased release of NETs and circulating markers of NETs (i.e. elastase) with high glucose (25mM) compared to low glucose (5mM) concentrations (54). The results of the present study are similar to the observations made by Joshi et al and Menegazzo L. In this study concentrations of glucose used were in the range of 5.5mM to 22mM with incubation time ranging from 30 minutes to 120 minutes. Results showed that there was concentration and time dependent increase in the elastase activity. The finding of increased elastase activity with increasing glucose concentrations support for the validation and the hypothesis that hyperglycemia activates neutrophils resulting in excessive release of elastase. The decrease in elastase activity observed after 120 minutes of incubation at all concentrations of glucose could be attributed to autolysis of elastase in the medium.

Neutrophil extracellular traps (NETs) are networks of extracellular fibril matrix of histones and granular proteins bound chromatin expelled from neutrophils. The process of formation of NETs is called as NETosis. The protein component of NETs include elastase, myeloperoxidase, cathepsin G, bactericidal permeability increasing protein and others bound to DNA backbone. Elastase is one of the main components of NETs. NETs can either fight disease or cause disease. Neutrophils, in addition to various mechanisms such as phagocytosis and release of antimicrobial factors, are known to eliminate pathogens by producing NETs. NETs have been also shown to induce host tissue damage associated with autoinflammatory diseases, preeclampsia, acute pancreatitis, Alzheimer’s disease and cancer metastasis (11, 55, 217).
High plasma homocysteine concentration is considered as an independent risk factor for developing cardiovascular diseases (56, 57). Since elastase also plays a key role in progression of inflammatory processes in cardiovascular diseases, this study was done to establish a relationship between elevated Hcys levels and elastase activity. The results showed that the activity of elastase released from freshly isolated neutrophils gradually increased with time and increasing concentrations of Hcys. After reaching a maximum activity at 120 minutes of incubation further increase of Hcys concentrations led to the inhibition of elastase release. These findings are in agreement with a study conducted by Joshi et al which showed increased release of NETs and associated elastase activity from neutrophils isolated from peripheral blood of healthy donors on increasing concentrations of Hcys. The authors also observed that in presence of Hcys, glucose primed neutrophils respond additively to produce NETs suggesting an association between hyperhomocysteinemia and diabetes (55). The present study results are in agreement with the earlier studies and supports that both hyperglycemia and hyperhomocysteinemia are inducers of release of elastase from neutrophils and could bring in detrimental effects leading to progressive disease conditions.

**Conclusion**

Higher concentrations of glucose and homocysteine increase release of elastase from neutrophils in vitro. The findings demonstrate that risk factors of diabetes and stroke activates neutrophils and the release of elastase. Thus elevated levels of neutrophil elastase could be an important determinant in the vascular complications of diabetes and stroke.