CHAPTER 2: INFLUENCE OF HYPER CHOLESTEROL ON AGGREGATION PARAMETERS OF ERYTHROCYTES IN TYPE II DIABETES MELLITUS.

2.1 Introduction

Diabetes Mellitus (DM) is a clinical term denoting a group of metabolic disorders in which impaired glucose utilization induces hyperglycemia. Carbohydrate, fat, and protein metabolism are all commonly deranged. The DM is a disease associated with a confusing array of metabolic abnormalities, which can also concern erythrocyte function. Diabetes is one of the most chronic illnesses of great economic and social importance. For every known diabetic there is another individual with an undiagnosed elevation of the blood glucose rendering him or her at risk of diabetic complication. According to Ganda and Soeldner, Diabetes mellitus is not a single disease entity, but a heterogeneous group of disorders with a striking diversity of etiopathogenetic mechanism and clinical manifestations. This is widely regarded as disorder associated with a relative or absolute deficiency of insulin, leading to impairment of glucose utilization mechanism, which is used not only to diagnose the condition but also to monitor the progress and control. These patients suffer from both macro- and microcirculatory complications. Type I or juvenile diabetes is characterized by extensive or total insulin deficiency. The patients usually develop this disorder under the age of 40 and require daily injections of insulin to survive. Type II or maturity onset diabetes occurs in the middle or later life. It is the most common type of disease and patients are treated with diet alone or diet and tablet as the
insulin secretion is delayed. Many changes are occurred in small and large blood vessels due to diabetes. The capillary endothelial cells themselves function abnormally.

**Hemorheological Changes**

Many hemorheological changes (viz., whole-blood, plasma and serum hyper viscosity, reduced erythrocyte deformability, increased red cell aggregation, and hyper-fibrinogenemia) are reported in a wide variety of clinical conditions and hemorheological disorders. These can contribute to cerebro-vascular complications and brain angiopathy by worsening cerebral blood flow and cerebro-vascular hemodynamic. There are increased frequencies of neuropathological and circulatory changes in diabetes. In the peripheral circulation, atherosclerosis frequently occur in arteries below the knee and the occurrence of gangrene of the lower limb is estimated to be forty times more common in diabetes compared to non diabetics. The major pathological event in the eye in these patients is the capillary non perfusion. Over a sustained time period the increasing number of occluded capillaries led to foci of retinal hypoxia finally leading to impairment or loss of vision. Clinical cardiac diseases and cardiac mortality are more frequent in diabetes. Disability and death from cerebro-vascular disease appears more common in diabetes especially brain infarction. The tissue structural changes are the result of complex interactions of hemorheological and biochemical factors (Stoltz et al, 1999).
Ditzel has demonstrated an abnormal blood rheology in diabetic microangiopathy. In diabetic patients red cell aggregation can be observed in the retinal and conjunctival venules and even arterioles, where normally high shear condition would cause the red cell to flow in mono dispersed state. The enhanced red cell aggregation is attributable partially to the change in flow condition resulting from vascular derangements and partially to elevations of alpha and beta globulins. The abnormal plasma protein pattern is probably the underlying cause for the increase of plasma viscosity and blood viscosity in diabetic adults and children. In diabetic ketoacidosis, blood viscosity may be further increased due to a reduction of cell deformability at low pH. The diabetic patients also show an increase in platelet adhesiveness (Stoltz et al. 1999).

**Induced changes in erythrocytes**

Diabetes mellitus, being a metabolic disorder, affects the functioning of the erythrocytes through interaction with its membrane and intracellular constituents. Some changes are associated with the impairment of glucose utilization process, whereas, others are induced by the dysfunctional mechanisms due to disease process, thus affecting the erythrocytes properties (Discher, 2000). Some of these changes which directly or indirectly affect the functional characteristics of erythrocytes are given below:

In diabetic patients serum and intra-erythrocyte sodium and serum potassium levels are increased significantly as compared to control subjects. The (Na+/K+)ATPase levels are significantly decreased which may cause disturbance of intracellular ionic balance and thus acceleration of cellular ageing (Gürbilek et
Magnesium in the cell is largely associated with ATP, as the complex Mg-ATP. ATP is less stable when it is not complexes with magnesium, so the loss of magnesium makes the cell more susceptible to stress, leading to an increased uptake of Ca2+ and diminished Ca2+-ATPase activity in comparison to healthy individuals. (Gonzalez Flecha et al, 1993).

The electronic spin resonance, using spin-labeled fatty acids, has shown structural changes at a depth of 0.6-0.8 nm from the membrane surface in the lipid bilayer of diabetic erythrocytes (Simpson, 1992). The membrane cholesterol is increased but there is four fold increase in phospholipids concentration in the membrane leading to a highly significant decrease in the ratio of cholesterol to phospholipids (Mawatari et al, 2004, Maksina et al, 1992).

Spectrin and actin are the two main structural proteins that together form a sub-membranous cytoskeleton meshwork that is responsible for the viscoelastic properties of the erythrocyte membrane. The spectrin-actin network combined with protein 4.1, which provides erythrocyte membrane the ability to withstand the stresses of circulation, has its origins in various levels of structural organization ((picart et al, 2000). The labeling of erythrocyte membranes with [3H]-borohydride, which labels glucose residues bound to proteins, revealed that several proteins are heavily glycosylated compared with non diabetic membrane. In particular, the proteins beta-spectrin, ankyrin, and protein 4.2 are the most glycosylated while the spectrin is oxidatively damaged (Schwartz et al, 1991).

Oxidative stress and increased insulin production contribute to endoplasmic reticulum stress, protein misfolding and induction of the unfolded
protein response, leading to pathological protein (Hayden et al, 2005, Pekpak et al 1999). The oxidative stress may further induce erythrocytes shape changes as observed under in vitro conditions by incubation of erythrocytes with H$_2$O$_2$ or ascorbate/Fe$^{2+}$, which transform discocytes to echinocytes due to involvement of membrane proteins (Hayden et al, 2005)

The changes that affect the respiratory function of the erythrocyte are reflected upon every tissue of the body. Erythrocytes of these patients have altered membrane dynamic properties and increased membrane thermo stability. It has also been reported that diabetic patients with poor metabolic control have lower erythrocyte membrane enzymes activity as compared to healthy control subjects. Diabetes is an important risk factor, which causes various diseases. Several investigations are going on all over the world to control the blood glucose levels, which is the major risk factor for various chronic complications. DM produces circulatory complications due to alteration in hemorheological profile. In more than 85% of the patients the DM is associated with vascular complications. Macroscopic properties such as plasma and blood viscosity are increased. The increased blood viscosity in diabetes reflects both reduced erythrocyte deformability and plasma protein changes that favors red cell aggregation. The sedimentation rate is elevated and 20-40% elevation of fibrinogen has been detected in diabetic blood (Macmillan, 1992). The combination of reduced red cell deformability and increased red cell aggregation in diabetes is the basis for a hemorheological model of the pathogenesis of diabetic microangiopathy. Greater red cell aggregation is the major cause of increased low shear rate blood
viscosity and elevated blood sedimentation rate. It is produced by high fibrinogen and haptaglobin levels that are crosssectionally linked to increase viscosity and to diabetic complications (Macmillan, 1992). The reduced deformability of diabetic erythrocytes is clearly a candidate for generating micro vascular damage. Red cells of diabetic patients have an altered membrane phospholipids asymmetry and an increased tendency to adhere to endothelial cells (Wali et al, 1988). Since a modification in phospholipids asymmetry influences the adhesion of erythrocytes to endothelial cells, it may be also involved in red cell aggregation by altering ability of erythrocytes to deform in to rouleaux, particularly through a modification in membrane lipid fluidity. Several changes in the erythrocyte function and structure have been observed in diabetes.

The major function of erythrocytes is to deliver oxygen from the lung to various tissues. Many studied have documented abnormalities of the oxygen carrying system in diabetes (Ditzet, 1979). Glycation reactions occur at different levels in the erythrocyte. The glycation of hemoglobin A1c (HbA1c) involves the binding site of 2-3DPG (glycolytic metabolite), HbA1c loses its ability to respond to changes in the concentration of this metabolite. The determination of HbA1c level is also used as a marker of the glycemic control. Due to their localization the erythrocyte membrane proteins, in contact with the hyperglycemic milieu, are highly glycated as a function of time (but also on the glycation sites number and accessibility on the molecules). This chemical reaction leads to the formation of advanced glycosylation end products (AGEs) which are involved in abnormal
adherence between erythrocytes and endothelial cells via a specific receptor (RAGE) (Wautier et al, 1991). A similar receptor is present on macrophages and could explain the reduced survival of erythrocytes in diabetes (Vlassara et al, 1987). The erythrocytes isolated from diabetic patients have an increased tendency to adhere to human umbilical vein endothelial cells (involving an intrinsic erythrocyte abnormality) and that it was correlated with the severity of the vascular complications (Wautier et al, 1991). This abnormal interaction between erythrocytes and endothelial cells had consequences on the arachidonic acid metabolism. This observation indicates that endothelium may be damaged by these abnormal interactions. Reduced membrane fluidity has been reported as a result of increased glycation of membrane proteins, either from diabetic patients, or from non diabetic humans after incubation of their erythrocytes with high glucose concentration in vitro (Watala et al, 1985). Red cells from diabetic patients aggregate more readily than those from normal subject (Ziegler et al, 1992). Immune mechanism of atherosclerosis in diabetes leads to the formation of LDL-ICs. These LDL-ICs are taken up by the macrophages and induce intracellular accumulation of cholesterol esters and increase in LDL-receptor expression. Erythrocyte aggregation in diabetes is favored by reduced negative surface charge. The surface sialic acid is responsible for 80% of the charge. This decrease has been linked to degree of hyperglycemia (Gandhi et al, 1979). Due to the enhanced aggregation in diabetes it leads to many complications in microcirculation like coronary heart disease, cerebral infarction and peripheral heart disease.
Diabetes-associated hyperglycemia causes protein glycation, also known as non-enzymatic glycosylation, which can adversely affect protein function (E.Y. Zhang et al, 1999), membrane protein glycation could determine a reduction of erythrocyte deformability and affecting the viscoelastic properties of erythrocytes (Lapolla et al, 1991) and an increase in the erythrocyte internal viscosity attributable to glycation-derived structural alterations in hemoglobin molecules (Watala et al, 1992). The increased intracellular viscosity of diabetic erythrocytes to higher levels of glycated hemoglobin would lead to the reduction in red blood cell deformability. (Paulisen et al, 1976).

Hypercholesterolemia (high blood cholesterol) is the presence of high levels of cholesterol in the blood. It is a metabolic derangement that can be secondary to many diseases and can contribute to many forms of disease, most notably cardiovascular disease. Elevated cholesterol in the blood is due to abnormalities in the levels of lipoproteins, the particles that carry cholesterol in the bloodstream. This may be related to diet, genetic factors (such as LDL receptor mutations in familial hypercholesterolemia) and the presence of other diseases such as diabetes and an under active thyroid. The type of hypercholesterolemia depends on which type of particle (such as low density lipoprotein) is present in excess. This is also increased emphasis on other risk factors for cardiovascular disease, such as high blood pressure. Longstanding elevated hypercholesterolemia leads to accelerated atherosclerosis; this can express itself in a number of cardiovascular diseases: coronary artery disease
(angina pectoris, heart attacks), stroke and short stroke-like episodes and peripheral vascular disease.

Hypercholesterolemia process induces changes in the erythrocyte membrane. This is primarily attributed to accumulation of cholesterol in the membrane (Vaya et al, 1996). The erythrocyte membrane consists of two domains, the lipid bilayer and the cytoskeleton (Smith, 1987). Phospholipids and cholesterol compose most of the lipid bilayer. Cholesterol is distributed evenly throughout the lipid domain, which alters flexibility and provides stability to the membrane. Any changes in cholesterol content of erythrocyte membrane changes the membrane property that alters the normal hemorheological properties like aggregation, deformability and shape etc. The aggregation, deformability and shape of the erythrocytes alter in both diabetes mellitus (Babu et al, 2004 & 2005; Sehyun Shin et al, 2007) and hyper cholesterol subjects (Manjunatha et al, 2000; Ercan et al, 2002) individually. The combined effect of both glucose and cholesterol makes complex changes in hemorheological parameters (Ercan et al, 2002). There are several in vivo mechanisms, which induce changes in erythrocyte membrane lipids and proteins and their interior, leading to alteration in erythrocyte shape, which in turn affects the deformability (Bessis et al, 1973; Marchesi, 1983; Lowe, 1988; Chien, 1987; Stoltz et all, 1999).

These studies show that hyperglycemia and hypercholesterolemia affect the hemorheological and morphological characteristics of erythrocytes. The
objective of the present work is to determine the aggregation parameters of erythrocytes in blood samples of diabetic patients, with normal cholesterol and hyper cholesterol concentration and is compared with healthy subjects to study the influence of cholesterol in diabetes mellitus on aggregation parameters of erythrocytes. There are various techniques used to measure the aggregation in various experimental and diseased conditions like malaria (Jayavanth et al, 2004), jaundice [Singh et al, 1995] and blood storage (Nagaprasad et al, 1998). In this study online aggregometer is used for aggregation measurements.

2.2 Materials and methods

2.2.1 Experimental techniques:

Erythrocyte Aggregometer.

Figure 2.1 shows block diagram of erythrocyte aggregometer. This is based on the principles of the attenuation of transmitted intensity of laser light due to scattering by erythrocytes and their aggregates after passing through the specimen chamber contains erythrocyte suspension in plasma at 5% hematocrit in the path of beam.

This consists of the following components.

2.2.1.1 Optical arrangement

The diameter of the He-Ne laser beam of wavelength 632.8nm is 1mm and power is 2mW (Spectra physics, U.S.A) is guided to the specimen chamber by reflection through two 45 degrees front-coated prisms. The beam is passed
through the center of the specimen chamber and the transmitted intensity varies depending on the size of the aggregates.

![Diagram of He-Ne laser aggregometer](Image)

**Figure 2.1.** Schematic diagram of He-Ne laser aggregometer.

### 2.2.1.2 Specimen chamber

The chamber is made of optically flat glass. The internal dimensions of the chamber are 8.0 x 1.6 x 70 mm. The erythrocyte suspension is placed up to a height of 60mm in the chamber.
2.2.1.3 Photo-detector assembly

The transmitted intensity after passing through the erythrocyte suspension is detected by a photo diode amplifier (HAD 1000 A; EG&G, USA). This detector assembly is selected due to its high speed, high sensitivity, low noise with high linear features. The detector is mounted on an amplifier/buffer assembly with its own power supply. The zero of the photo diode is adjusted using an offset circuitry under dark conditions. The maximum of the assembly is calibrated to +10V output for 100% transmission by placing plasma only in the specimen chamber. The detector output is connected to a PC through an ADC interface of full-scale input of ±5V via a 2:1 potential divider.

2.2.1.4 ADC interface

The interface is a 12 bit analog to digital converter (AD574, Analog devices USA). The A/D conversion is of the successive approximation type with a conversion time about 25 microseconds. The A/D conversion has a built in 8-channel multiplexer but only one channel is used for this system.

2.2.1.5 Display

The output from the ADC is then fed to the PC, where a data file is open to store this digitized output. For further analysis and display of the signal the data file is accessed and the various parameters from the signal are obtained by the software developed indigenously.
2.2.2 Principle

The laser aggregometer is based on the principle of the attenuation of the transmitted intensity (TI) due to scattering by erythrocytes and their aggregates in the beam path. Initially the sample being well mixed, the erythrocytes are in a monodispersed condition and the population of cells in the observed volume (OV) is maximum, hence they scatter the light effectively and attenuate the TI rendering it to a minimum value and due to movement of the formed aggregates in observation volume (OV) during sedimentation process, the TI would have fluctuations overriding it, which are characteristic of the size of aggregates. Figure 2.2 shows variation of transmitted intensity obtained during aggregation process.

The transmitted light intensity after passing through the suspension is given by

\[ I_t = I_o . e^{-\alpha L} \]  (1)

where \( I_o \) = TI of laser with only plasma in the chamber,
\( \alpha \) = Attenuation coefficient of the suspension,
\( L \) = Path length offered by the erythrocytes and it is given by

\[ L = N_t \cdot x \]  (2)

\( N_t \) = Number of erythrocyte in the observation volume (OV) at time t
\( x \) = Mean thickness of erythrocyte (4 micron).

From equation (1) and (2)

\[ N_t = \frac{1}{\alpha . x} \ln \left( \frac{I_o}{I_t} \right) \]  (3)
Attenuation coefficient, is determined using the equation (1) at the beginning of the Process at $t = 0$ which will then be

$$I_{t0} = I_0 e^{-\alpha N_0 x} \quad (4)$$

For this TI is measured by placing a well-mixed sample in the chamber to give $I_{t0}$ at $t=0$.

The number of monodispersed cells in the OV at $t=0$ ($N_0$) is calculated by considering the OV to be a cylindrical (1mm diameter and 1.6mm length) based on $5 \times 10^6$ cells /cu.mm for 5% hct. Substituting $I_{t0}$, $N_0$ and $I_0$, $\alpha$ works out to be
By substituting the values of $\alpha$, $x$, $I_o$ and $I_t$, the number of cells $N_t$ at any instant of time can be calculated using the equation (3).

The TI is associated with fluctuation characterized by peaks and troughs. Analysis of TI for $N_t$ at the peak of a given fluctuation provides minimum number of cells ($N_{t_{\text{min}}}$) and at the trough provides the maximum number of cells ($N_{t_{\text{max}}}$). The variation in the number of cells ($N_{t_{\text{max}}} - N_{t_{\text{min}}}$) is indicative of the change in aggregate size. To minimize the contribution of noisy fluctuations to the results, change in ($N_{t_{\text{max}}} - N_{t_{\text{min}}}$) is thresholded at 3000 and remaining fluctuations are classified into four categories. Figure 2.3 shows the flow chart used for the measurement of aggregation parameters.

2.2.3 Aggregation parameters.

2.2.3.1 Process initiation time (PIT).

It is the time in minutes taken for the appearance of fluctuation in TI.

2.2.3.2 Process completion time (PCT).

It indicates the total time in minutes required for the completion of the sedimentation from the beginning till the TI reaches the intensity ($I_0$) when plasma alone in the OV.

2.2.3.3 Aggregation size index (ASI).

It indicates the instantaneous change in the size of the aggregates given by the expression $N_{t_{\text{max}}} - N_{t_{\text{min}}}$.

Where $N_{t_{\text{max}}}$ - number of cells at trough of the fluctuation of TI at time $t$.

$N_{t_{\text{min}}}$ - number of cells at peak of the fluctuation of TI at time $t$.

Aggregates are divided into four categories viz depending upon their sizes.
Figure 2.3. Flow chart for measurement of aggregation parameters
2.2.3.4. Effective number of cells (ENC).

It represents the effective number of cells in the observed volume (OV) at any given time and is derived from ASI.

\[
ENC = N_{t_{\text{max}}} - \frac{\sum_{i=1}^{k} n_i N_i}{\sum_{i=1}^{k} n_i}
\]

Where \( n_i \) - number of fluctuations in \( i^{th} \) class

\( N_i \) - average number of cells in \( i^{th} \) class.

2.2.3.5. Effective cellular sedimentation duration (ECSD).

It is an indicative of the effective time duration for the formed aggregates to sediment through observed volume.

\[
ECSD = \frac{\sum_{i=1}^{k} n_i - T_i}{\sum_{i=1}^{k} n_i}
\]

Where \( T_i \) = mean sedimentation time of the \( i^{th} \) class.

2.3 Sample preparation.

Fresh blood samples were collected in the morning before food, from healthy subjects with glucose concentrations 90 to 120 mg% (\( n = 10 \)), diabetes mellitus patients with glucose concentrations 210 to 260 mg% (glycosylated hemoglobin 9 to 10%) with normal cholesterol 130 to 230 mg/dl (Group A, \( n=24 \)) and diabetes mellitus with same glucose level as group A with hyper cholesterol of more than 280 mg/dl (Group B, \( n=21 \)) by venepuncture in test tube containing citrate phosphate dextrose (10: 1.4), as an anticoagulant. Each blood sample was centrifuged at 2000g for 30 min. The supernatant plasma was separated and the buffy coat on the top of cells was separated and discarded. The suspensions of
5% hematocrit were prepared in plasma. Healthy subjects selected for the study had no clinical disease and their plasma and serum biochemical levels are within normal range. Their systolic and diastolic blood pressures were within normal range and hematocrit within 36 to 45%. The age of healthy and diabetic subjects ranged from 30–60 years. These measurements were carried out in a room maintained at temperature 25 ± 1 Centigrade. The statistical analysis of the data was carried out by Student t-test.

2.4 Data collection and processing

The specimen chamber filled with well-mixed erythrocyte suspension was placed between source of laser beam and detector. The erythrocyte aggregates formed in the chamber, sediment under gravitational field and pass through the observed volume. The transmitted intensity (TI) varies depending on the size of the aggregates present in the path of the laser light beam. These data’s are collected at a sampling rate of 5 samples per second sequentially till the beam attains maximum value that is same as plasma alone present in the chamber. This transmitted intensity was digitized and stored for further analysis.

2.5 Results

The figure 2.4 shows the variation of transmitted intensity (TI) of erythrocyte suspension of a normal, group A and group B samples. Initially there is no significant change in TI is found for nearly 5 minutes. After that there is gradual increase of TI with large fluctuations in amplitude and attains a maximum value. This is because large number of erythrocytes and their formed aggregates in the observed volume makes large scattering of light and hence minimum transmitted
intensity. The amplitude of the fluctuations varies, indicating the change in aggregate size that crossed the observed volume due to gravity. Finally, due to sedimentation of formed aggregates through the observed volume there is no fluctuations in the transmitted intensity were found, indicating that all the formed aggregates have crossed the observed volume and shows a steady constant value in TI. The transmitted intensity is reached the steady constant value fastest in group B and faster in group A comparing to normal. The TI is associated with fluctuation characterized by peaks and troughs. Analysis of TI for \( N_t \) at the peak of a given fluctuation provides minimum number of cells \( (N_t_{\text{min}}) \) and at the trough provides the maximum number of cells \( (N_t_{\text{max}}) \). The variation in the number of cells \( (N_t_{\text{max}} - N_t_{\text{min}}) \) is indicative of the change in aggregate size. To minimize the contribution of noisy fluctuations to the results, change in \( (N_t_{\text{max}} - N_t_{\text{min}}) \) is thresholded at 3000 and remaining fluctuations are classified into four categories of various sizes based on the TI data’s. The categories are the aggregates having the cells of (3000-3500), (3501-5000), (5001-10,000), and above 10,000.

Figure 2.5 (a) shows change in ASI of four categories of normal subjects in four minutes interval. Initially large numbers of aggregates are found in each category of first 4 minutes interval, and gradually decrease in each category with respect to next 4 minutes intervals of time and so on for normal samples. The pattern of aggregate size index is altered in group A (Fig. 2.5 (b)) and group B (Fig. 2.5 (c)) of diabetes samples. The sedimentation pattern of different size aggregates varies. Aggregates of various sizes in normal samples sediment slowly but these are faster in group A and fastest in group B due to the formation
of larger size aggregates. The changes in patterns of ASI in group A are due to higher blood glucose levels and in group B, the more changes in pattern was observed is due to the effect of hyper cholesterol.

![Graph showing variation of transmitted intensity (TI) in normal, group A [G1] and group B [G2] subjects, with respect to time.](image)

**Figure 2.4.** Variation of transmitted intensity (TI) in normal, group A [G1] and group B [G2] subjects, with respect to time.

The figure 2.6 shows a comparison of the effective number of cells (ENC) in observed volume (OV) at various intervals of time in the path of the laser beam. Initially due to well-mixed sample having same hematocrit in observed volume, the value of ENC is same for normal, group A and group B of diabetes mellitus. As time increases, nearly after 12 minutes, the value of ENC is less for group A and much less for group B comparing to control subjects throughout the sedimentation process. This is due to large aggregates forms in group B sediment passed through the observed volume quicker than group A and control.
Figure 2.7 shows the variation of the effective cellular sedimentation duration (ECSD) of the normal, group A and group B. This data is an indicator of effective time taken by the aggregates to pass through the observed volume. The aggregates of normal samples take longer duration to pass through the observed volume comparing to group A and group B. The ECSD in group A is less than normal indicates the formed aggregates in group A sediments quicker than normal due to influence of blood glucose and in group B have further less value than group A due to combined effect of cholesterol and glucose.

Table 2.1 shows the comparison of the aggregation parameters PIT, PCT, ENC and ECSD. The values of above aggregation parameters are decreased in group A and further decreased in group B was found compare to control indicating more aggregation in group B than group A comparing to control subjects. The data’s of ENC and ECSD are chosen corresponding to 14th minute. The elevated plasma fibrinogen in group A and further elevated in group B supports the enhanced aggregation.

Table 2.1. Comparison of aggregation parameters (Mean ± SD) of normal, group A and group B subjects

<table>
<thead>
<tr>
<th>Erythrocytes</th>
<th>PIT (min)</th>
<th>PCT (min)</th>
<th>ENC (14th min)</th>
<th>ECSD (14th min) (sec)</th>
<th>Fibrinogen mg/dl</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>1.25 ± 0.23</td>
<td>27.25 ± 5.03</td>
<td>434200 ± 80600</td>
<td>0.41 ± 0.09</td>
<td>272 ± 5.5</td>
</tr>
<tr>
<td>Group 1</td>
<td>1.18 ± 0.26*</td>
<td>24.08 ± 3.02*</td>
<td>331500 ± 12500^</td>
<td>0.39 ± 0.08^</td>
<td>406 ± 13**</td>
</tr>
<tr>
<td>Group 2</td>
<td>1.02 ± 0.24*</td>
<td>16.23 ± 2.4**</td>
<td>101900 ± 6300**</td>
<td>0.35 ± 0.04*</td>
<td>504 ± 21**</td>
</tr>
</tbody>
</table>

Compared with the normal, * p < 0.05; ^ p < 0.005; ** p < 0.0004
Figure 2.5. Comparison of aggregation size index (ASI) of normal (a), group A (b) and Group B (c) subjects.
Figure 2.6. Comparison of effective number of cells (ENC) of normal, group A (G1) and group B (G2) subjects

2.6 Discussion

Aggregation of erythrocytes of control and hyperglycemic subjects with normal and hyper cholesterol were analyzed by online aggregometer. The significant elevation of aggregation of erythrocytes was found in diabetes with hyper cholesterol than diabetes with normal cholesterol comparing to control subjects. The extent of RBC aggregation is determined by opposing forces: the repulsive force between the negatively charged cells, the cell-to-cell adhesion induced by plasma proteins (Nash et al, 1987). The process of aggregation of erythrocytes is due to presence of plasma protein such as fibrinogen and it was elevated in diabetes mellitus due to hyperglycemia (Mcmillan, 1992) and
cholesterol (Ercan et al, 2002), which leads hyper aggregation. The availability of enhanced concentration of haptoglobin further mediates this process.

![Figure 2.7. Comparison of effective cellular sedimentation duration (ECSD) of normal, group A (G1) and group B (G2) subjects](image)

Erythrocyte membrane surface charge and membrane mechanical behavior are the most commonly considered biophysical properties of RBC vis-à-vis their aggregation behavior (Chien et al, 1990, Meiselman, 1993). Reports of RBC aggregation behavior in diabetes mellitus are not always in concordance, possibly due to differing patient characteristics or degree of metabolic control. There are cellular factors affecting RBC aggregability that vary between
individuals, between cell fractions from a given individual, and with various pathological states.

In poorly controlled type 2 diabetes, RBC aggregation in plasma is significantly elevated, yet no differences from control were noted for cells washed and re-suspended in a 1% isotonic solution of 110 kDa dextran (Bauersachs, et al, 1989). Pathological RBC aggregation is characterized by strong intercellular interactions and may affect flow dynamics, mainly within the microcirculation, where shear stress is low. The vessels most likely to be affected by RBC aggregation are the postcapillary venules, where vessel diameter is large enough and shear stress is low enough to allow aggregation.

The cause of increased in aggregation is due to decrease in sialic acid (Gandhi et al,1979) content of glycoporin A in diabetic’s erythrocyte membrane, alteration in the membrane lipid composition and increase in glycosylated hemoglobin (HbA1c). These changes will be common for both hyperglycemia and hypercholesterolemia. Such cells due to their altered properties may not able to carry out tank-tread motion while flowing through micro vessels (Fischer et al, 1978).

Our observations on increased sedimentation of erythrocytes in diabetes with hyper cholesterol supports the formation of large aggregates as these settle down faster in plasma compared to that of normal cholesterol and control subjects. The decrease in effective number of cells (ENC) in observed volume, and decrease in process completion time (PCT) supports this statement. The aggregate size index, a parameter related to the size of aggregate, increases
with the increase of glucose concentration and further increases with increase of hyper cholesterol. The higher aggregation tendency leads to formations of larger and stronger aggregates that may not dis-aggregate, particularly in diseased conditions.

In conclusion the hyperglycemia and hypercholesterolemia produces significant changes in erythrocytes. These factors combined with increased tendency of adhesiveness and elevated levels of fibrinogen lead to increased erythrocytes aggregation. This elevated aggregation is due to additional effect of hypercholesterolemia in hyperglycemic subjects. This contributory factor in microcirculatory complications as observed in diabetic patients is more severe in presence of hyper cholesterol.