Renal function declines rapidly into ESRD in uncontrolled DM [412,413]. Hemodynamic and other physiological changes that occur during the onset and progression of DN have been fairly well characterized [414]. As a result of lifestyle changes and ageing of the population, DM is becoming one of the most important worldwide public health problems [415]. Some recent epidemiological studies, mostly performed on African and Asian populations, have also reported that type 2 diabetic patients are characterized by increased erythrocyte osmotic fragility[416-418].

Cholesterol is essential for maintenance of the structural and functional integrity of the biological membranes. It is also involved in the activity of membrane bound enzymes [419]. Alterations in membrane fluidity are determined by the amount of cholesterol and cholesterol/phospholipid molar ratio [420]. The observed increase in cholesterol and c/p ratio indicates the loss of membrane fluidity in DN [420]. Alterations in the erythrocyte lipid composition may be a reflection of altered plasma lipid, due to an ineffective exchange mechanism with plasma. Diabetes is a metabolic syndrome characterized by hyperglycemia resulting from alterations of insulin secretion or action. Constant exposure to free radicals and high oxidative stress in diabetes have also been associated with erythrocyte structural damage [421]. Lipid peroxidation alters the cellular structure of membrane-bound enzymes by changing phospholipids and fatty acid composition.

Na⁺K⁺-ATPase is the major force of sodium transport in proximal tubular cells and as an ion transporter it is only active when inserted in its physiological place in
the basal membrane [422]. It involved in trans membrane cation regulation via ATP–
dependent dual efflux/influx of sodium (Na+) and potassium (K+) ions in various cells
[423,424]. The regulation of this pump activity is dependent on the phosphorylation
of the α-subunit of Na⁺K⁺-ATPase [423,424]. In the kidney ANGII blocks this
translocation of Na⁺K⁺-ATPase leading to dysfunctional enzyme activity [426].
Recently demonstrated also in STZ-diabetic ras that the renal Na⁺K⁺-ATPase is
mislocated from the tubular basal membrane toward the cytoplasm and thus becomes
non-functional. Exogenous ANGII administration led to further impairment of Na⁺K⁺-
ATPase and superimposed progression of DN [226, 427].

The non-nucleated erythrocyte is unique among human cells, an important and
distinguishing feature of the discoid human red cell is its ability to undergo large
passive deformations during repeated passage through the narrow capillaries of the
microvasculature, with cross-sections one-third its own diameter, throughout its 120-
day life span. Therefore cellular deformability and membrane integrity plays a key
role for in regulating red cell function and survival [428].

Due to arrival of altered levels of biochemical and tissue products in the blood
and their interactions with blood constituents the functional properties of erythrocytes
are changed, which in turn affect their hemorheological, i.e., blood flow properties
under in vivo and in vitro conditions. At cellular levels the parameters which are
affected include the aggregation and deformability of erythrocytes. [429]. The shape
transformation of erythrocytes while flowing through micro-vessels makes major
contributions to flow resistance and is directly related to the erythrocyte deformability
under in vitro and in vivo conditions [430]. In combination with plasma proteins
fibrinogen and globulins, the aggregation of erythrocytes, a reversible phenomenon related to formation of three-dimensional chain like structure, take place [431].

Since chronic kidney disease due to DN is becoming an ever larger health burden worldwide more effective therapies are desperately needed. So the present study designed to find the efficacy of NARN on structural integrity of red blood cells in STZ-induced DN by measuring the osmotic fragility and permeability of erythrocytes.

10.1 RESULTS

Table 10.1 and figure 10.1 show the activity of membrane bound Na⁺K⁺-ATPase in control and experimental animals in each group. The activity of membrane bound Na⁺K⁺-ATPase was significantly decreased in RBC membrane of STZ-induced DN animals as compared to control group animals. The activity of Na⁺K⁺-ATPase was restored in NARN (50 mg/kg b.wt for 15 days) and AG (100 mg/kg b.wt) treated animals in group III and group IV respectively. No significant differences were noticed between control and animals treated with the NARN alone.

Osmotic fragility curves for control and experimental animals in each group are shown in figure 10.2. The fragility curve of STZ-induced DN animals was shifted to the right for the control animals. The mean corpuscular fragility was also significantly higher in STZ-induced DN (group II) animals as compared to controls (table 10.1 and figure 10.1). Treatment of STZ-induced DN animals with NARN and AG shifted the curve to the left, those of DN animals. Mean corpuscular fragility values did not differ significantly in animals treated with the NARN alone as compared to control animals.
Table 10.1 Effects of NARN on membrane bound enzyme (Na⁺K⁺-ATPase) and MCF in STZ-induced diabetic nephropathy rats

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Group I (Control)</th>
<th>Group II DN</th>
<th>Group III DN + NARN</th>
<th>Group IV DN + AG (Positive control)</th>
<th>Group V NARN</th>
</tr>
</thead>
<tbody>
<tr>
<td>RBC membrane Na⁺K⁺-ATPase*</td>
<td>0.42±0.03&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.21±0.04&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.37±0.02&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.39±0.03&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.41±0.03&lt;sup&gt;ad&lt;/sup&gt;</td>
</tr>
<tr>
<td>Mean Corpuscular Fragility**</td>
<td>0.43±0.031&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.60±0.032&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.43±0.027&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.44±0.026&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.41±0.02&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SD for 6 animals in each group; Values not sharing a common superscript letter differ significantly at p<0.05 (DMRT); DN- Diabetic Nephropathy; NARN- Naringenin; AG- Aminoguanidine; * μ moles of inorganic phosphorus liberated / hour / mg protein; **Concentration of NaCl solution (%) at 50% hemolysis
Figure 10.1: Effect of NARN on serum immunoglobulin in STZ induced diabetic nephropathy.

The values that do not share a common superscript letter (a, b, c and d) between groups differ significantly at p<0.05 (Analysis of variance followed by DMRT; n=10).* μ moles of inorganic phosphorus liberated / hour / mg protein; **Concentration of NaCl solution (%) at 50% hemolysis
Figure 10.2 Erythrocyte osmotic fragility curves for normal and experimental animals.
Photographs of RBC

Figure 10.3i-iii (A-J) shows the photomicrography of Red Blood Cells smears from blood samples of normal and experimental animals. In group I animal’s erythrocytes are shows within normal limits. There were no morphological changes. But in group II STZ-induced diabetic nephropathy animal’s erythrocyte showed an altered cell structure and formation of acanthocytes. The structure of erythrocytes in NARN and AG treated group of rats were found to be significantly normal. There are no morphological structural changes found in NARN alone treated group of animals (group V).
Figure 10.3i. Photomicrography of red blood cells smears from blood samples of normal and experimental animals (group I and II). Blood smears were prepared, dried. The morphology of red blood cells was evaluated under optical microscopy (40X) after image capture.
Figure 10.3ii. Photomicrography of Red blood cells smears from blood samples of group III and group IV animals. Blood smears were prepared, dried. The morphology of red blood cells was evaluated under optical microscopy (40X) after image capture.
Figure 10.3iii. Photomicrography of Red blood cells smears from blood samples of group V NARN alone treated animals. Blood smears were prepared, dried. The morphology of red blood cells was evaluated under optical microscopy (40X) after image capture.
10.2 DISCUSSIONS

The increased glucose concentration may affect the basic functions of erythrocytes, related to exchange of metabolic products at the systemic and pulmonary capillaries [429, 432] The biochemical alterations in plasma and erythrocyte in diabetes mellitus have direct influence on the hemorheological properties of cells. The chronic hyperglycemia in DM [433] leads to many hematological abnormalities, including alterations in erythrocyte membrane structure [433]. In the diabetes all the erythrocytes are not affected. The severity of change in erythrocyte shape depends on plasma glucose level [434]. The deformability and fluidic nature of the RBC’s is very important for the survival [435]. The reasons for formation of abnormal erythrocyte cell membranes include alteration of the ratio of cholesterol to phospholipid (chapter IX) table 9.2 in the core of the erythrocyte cell membrane in DN [436]. Also, \textit{de novo} oxidative damage, a result of increased glycosylation proteins namely, collagen, (figure 7.4)LDL (figure 9.2) and hemoglobin (figure 7.3) could participate in the mechanism, whereby diabetic erythrocytes may acquire membrane abnormalities of DN animals[437].

The shape parameters (i.e., projected area, perimeter and form factor) significantly differ between diabetic and non-diabetic erythrocytes[434]. Riquelme \textit{et al}[438] observed that glucose concentration as low as 1% induces a non-enzymatic glycosylation of cell surface proteins, which can modify the visco-elastic properties and decreases the dynamic elasticity of erythrocytes [438]. The fluidity of RBC membranes [439], as well as the deformability of erythrocytes was significantly decreased in a dose- and time-dependent manner when incubating RBCs in a high glucose medium [440]. A higher oxidative stress might also contribute to increase the
lipid peroxidation of RBC membranes and thereby enhance the fragility of erythrocytes in animals with DN [417]. Another potential factor contributing to modify the dynamic properties of erythrocytes is the increased glycosylation-derived internal viscosity, which is common place in animals with DN [441]. It is thereby conceivable that all of these hyperglycemia induced abnormalities would finally alter the dynamic proprieties of erythrocytes, increase their fragility and complicate the flow of erythrocytes through the microcirculation of group II animals.

The Na\(^+\)K\(^+\)-ATPase activity is low under resting conditions and can be regulated by several hormones, notably catecholamines and insulin [442]. Even though the stimulation elicited by hormones is only approximately 5% of the increase in Na\(^+\)K\(^+\)-ATPase activity caused by maximal electrical stimulation of muscle [443], hormonal regulation remains a key mechanism for the control of Na\(^+\) and K\(^+\) gradients. Stimulation of the Na\(^+\)K\(^+\)-ATPase by insulin has been documented in rat adipocytes, skeletal muscle, kidney, liver and in cells in culture such as hepatocytes [444]. The Na\(^+\)K\(^+\)-ATPase represents the only route of Na\(^+\) efflux from most mammalian cells and is the major path for K\(^+\) uptake from the blood. It thus establishes and maintains the low intracellular Na\(^+\) and high intracellular K\(^+\) concentration observed under physiological conditions [445].

Studies suggest that insulin plays a stimulatory role in Na\(^+\)K\(^+\)-ATPase activity through tyrosine phosphorylation process [423]. The relatively reduced levels of erythrocyte Na\(^+\)K\(^+\)-ATPase activity in diabetic nephropathy rats (table 10.1 and figure 10.1) was consistent with the findings of previous authors. Soulis-Liparota et al [446] reported reduced Na\(^+\)K\(^+\)-ATPase activity STZ-induced diabetic rats with nephropathy. Our findings reported here was in concordance with those of Konukoglu et al [447].
They noted that hypercholesterolemia and free radical-induced mechanisms may be responsible for the inhibition of erythrocyte Na$^+$/K$^+$-ATPase activity in animals with DN (group II). According to the present study, decreased erythrocyte Na$^+$/K$^+$-ATPase activity of hyperglycemic rats was analogous to altered enzyme activity in RBC of animals with DN. According to Greene et al [448], impaired Na$^+$/K$^+$-ATPase activity is induced by hyperglycemia with characteristic distortions in myo-inositol and phosphoinositol metabolism, which normalizes with intensive insulin therapy that controls hyperglycemia [449]. Thus, decreased erythrocyte Na$^+$/K$^+$-ATPase activity was an obvious confirmation of a connection between the capacity of erythrocyte to actively transport Na$^+$/K$^+$ ions (antiport) and obligatory utilization of ATP for α-subunit of Na$^+$/K$^+$-ATPase phosphorylation required for enzyme activity [423, 425, 450] of DN animals. Hyperglycemia with associated depressed glucose utilization in diabetic states results in low intracellular ATP concentration, insufficient for the required obligatory phosphorylation of the enzyme. The increase in erythrocyte Na$^+$/K$^+$-ATPase activity in DN rats treated with NARN (group III) as instrument of glycemic control was an indication of improve glucose utilization exemplified in DN rats treated with the standard Aminoguanidine (AG) drug (group IV). The role and mechanism of insulin in regulation of Na$^+$/K$^+$-ATPase activity has been described elsewhere [451]. In another study, Konukoglu et al., [447] reported that hypercholesterolemia and free radical induced mechanisms may be responsible for the inhibition of erythrocyte Na$^+$/K$^+$-ATPase activity patients with type 2 DM. The present study showed that erythrocyte Na$^+$/K$^+$-ATPase activities gave insights into the pathophysiology of diabetic state and could serve as a biomarker for ascertaining therapeutic control in DN.
We observed an altered activity of erythrocyte membrane bound enzyme (Na\(^+\)K\(^+\)-ATPase) and disturbed extracellular (Na\(^+\)) and intracellular (K\(^+\)) cation in the plasma (chapter 6; table 6.2) of DN rats which suggest that the membrane permeability is affected during STZ-induced DN. Free radical induced oxidative damage to membrane ATPase has been assumed to be crucial for cell lysis [452]. Serum and intra-erythrocyte sodium and serum potassium levels are increased significantly in DN rats as compared to control animals. The Na\(^+\)K\(^+\)-ATPase levels are significantly (p<0.05) decreased which may cause disturbance of intracellular ion balance and thereby acceleration of cellular ageing [453]. This further leads to an increase in cell size and osmotic fragility, which contribute to the disturbances in micro-vascular circulation observed in DN [454].

Erythrocytes and erythrocyte membrane are more vulnerable to lipid peroxidation due to constant exposure to high oxygen tension and richness in polyunsaturated fatty acids respectively [455]. Increased osmotic fragility (figure 10.2) in DN animals can be due to the increased oxidative stress in erythrocytes(table 8.2). Over production of ROS has been implicated in the alterations of membrane structure and function. Increased lipid peroxidation observed in present study (chapter 8) is therefore responsible for the increase in osmotic fragility [456] of DN animals. Several studies have demonstrated that GSH plays a central role in maintaining cellular integrity and membrane fragility [457]. Decline in red blood cell reduced glutathione (table 8.2) observed in DN animals is partly be responsible for the increased osmotic fragility of erythrocytes [457].

Membrane lipids constitute about 50% of the mass of most animal cell plasma membranes. They play an important role in determining the various function and
properties of red cells such as maintaining the integrity, permeability, fluidity, and function. Membrane fluidity is known to be dependent on the molar ratio of cholesterol to phospholipid [458] of DN animals. Measurement of Mean Corpuscular Fragility (MCF) of erythrocyte membranes is useful to assess the alterations in the integrity of cell structure and function [459]. Alteration in membrane fragility has been documented in hemolytic diseases, DM and DN [459]. This finding was in line with study conducted by Ibanga [416], where they found that the MCF was increased in patients with diabetes. Our study findings supports that indeed there is alteration of the membrane fragility in DN animals.

Cholesterol is essential for maintenance of the structural and functional integrity of the biological membranes. It is also involved in the activity of membrane bound enzymes [419]. Alterations in membrane fluidity are determined by the amount of cholesterol and cholesterol/phospholipid molar ratio (figure 9.3) of group II animals [420]. The observed increase in cholesterol and c/p ratio indicates the loss of membrane fluidity [420] in DN animals. Alterations in the erythrocyte lipid composition may be a reflection of altered plasma lipid, due to an ineffective exchange mechanism with plasma.

During in the cholesterol accumulation process the structure of the membrane is slowly changed. At low concentrations spicules were formed on the membrane. With increase in cholesterol acquire an echinocytic appearance [460, 461] in STZ-induced DN animals (figure 10.3 C and D). The observed biochemical changes also resulted in significant morphological changes in the erythrocytes of STZ-induced DN rats. Changes in membrane lipid composition lead to morphological changes, the prominent changes were the distortions in normal discocyte shape, appearance of
central and peripheral protuberances and formation of acanthocytes [462] in group II animals. The drug treatment (NARN) had potent therapeutic efficacy in modulating erythrocyte function and structural abnormalities by this remarkable hypocholesterolaemic and antioxidant property.

From this study, it is evident that glucose enhancement in plasma produces specific alterations in the erythrocyte membrane [463] of DN. Increased erythrocyte fragility and permeability in DN animals are probably due to their altered lipids, lipid peroxidation and antioxidant status. Oral administration of NARN to these DN animals prevented the alterations in red cell fragility and the activity of membrane bound Na⁺K⁺-ATPase, which indicates the role of NARN in maintaining the structural integrity of erythrocytes during DN. The mechanism for increased fragility could include increased glycosylation of the membrane protein, alteration of the Na⁺K⁺-ATPase on the erythrocyte membrane and there is a necessary to emphasize on further research to understand the mechanisms of exacerbated red cell fragility, in order to prevent the complications of DN.