Diabetic nephropathy (DN), a condition of progressive damage of the kidney, is the second most prevalent diabetes-associated complication inferior to cardiovascular disorders, eventually leading to end-stage renal failure [236,237]. The hallmark of DN includes deposition of ECM proteins such as, collagen, laminin and fibronectin in the mesangium and renal tubulo-interstitium of the glomerulus and basement membranes. Thus, the accumulation of ECM proteins plays a critical role in the development of DN. ECM synthesis and its expression in high glucoseambience \textit{in vitro} and \textit{in vivo} states.

Inflammation plays a pivotal role in the pathogenesis of DN. Inflammatory cytokines, primarily IL-1, IL-6, IL-18 and TNF-\(\alpha\) are involved in the development and progression of DN [238,239]. TNF-\(\alpha\) is one of the main pro-inflammatory cytokines and may be produced intrinsically in renal cells [240,241] and also secreted predominantly from monocytes and macrophages that is functional in lipid metabolism, coagulation, insulin resistance and endothelial biology.

TNF-\(\alpha\) mRNA is abundantly expressed in diabetic rats, it has been suggested that TNF-\(\alpha\) may contribute to the induction of nephropathy by stimulating albuminuria [242]. TNF-\(\alpha\) has been shown to stimulate the release of other chemokines and growth factors, including MCP-1 and TFG-\(\beta\) [243,244], suggesting the involvement of TNF-\(\alpha\) in renal fibrosis.

IL-1 increases the expression of chemotactic factors and adhesion molecules, enhances vascular endothelial permeability and stimulates the proliferation of mesangial cells and matrix synthesis [62, 245]. The elevated levels of IL-1 in kidneys
from male Sprague-Dawley rats treated with streptozotocin compared to control rats[246] and renal expression of IL-1 is significantly correlated with urinary albumin excretion [247].

Renal IL-6 expression is positively related to mesangial proliferation, tubular atrophy in diverse models of renal disease, supporting the role of IL-6 in the progression of renal disease [62]. Serum IL-6 levels are significantly higher in patients with type 2 diabetic nephropathy compared to levels observed in diabetic patients without nephropathy suggesting a role for IL-6 in the pathogenesis of DN [248,249]. In fact, serum IL-6 levels are similar in type 2 diabetic patients with normal albumin excretion and micro albuminuria, but significantly increased in patients with DN and clinical albuminuria [239]. Using high-resolution in situ hybridization in kidney biopsies of Japanese patients with DN, it was found that interstitial expression of IL-6 mRNA correlated significantly with the degree of interstitial injury [250]. Clinical reports are in agreement with studies in experimental animals where renal expression of IL-6 mRNA is increased in streptozotoc in diabetic rats compared to controls and levels are significantly associated with urinary albumin excretion [247]. These data support a role for IL-6 in the progression of DN in the later stages of the disease.

IL-18 is a potent inflammatory cytokine secreted from activate dmonocytes/macrophages and it is known to induce interferon-γ (IFN-γ), which increases functional chemokine receptor expression in human mesangial cells [245]. Expression of IL-18 is increased in renal biopsies from patients with DN in proximal and epithelial tubular cells [251] and patients with type 2 diabetes have significantly higher serum and urinary levels of IL-18 compared to healthy controls [239,249,252,253]. Moreover, there is a positive correlation between IL-18 levels in
diabetic patients and the development of urinary albumin excretion, with the highest IL-18 levels found in patients with microalbuminuria and clinical albuminuria [239,249,253,254].

Abnormal levels of immunoglobulins are very common in diabetic patients [255] and the probability that changes in immunoglobulin levels are implicated in the pathogenesis of infection requires further exploration. IgA has the highest concentrations in body’s fluids such as saliva and alsoin mucus covering the surface of intestine and respiratory tracts [256]. The glycosylation of the IgG significantly increases its vascular clearance rate [257]. The significant increase in vascular clearance of glycosylated IgG may thus play a significant role in DN.

Hemoglobin A1c (HbA1c), the most widely used assay, measures the percentage of circulating hemoglobin that has chemically reacted with glucose and reflects ambient blood glucose control over the prior 120 days, with the most profound effect in the preceding 30 days [258,259]. HbA1c may reflect a pathogenic mechanism of glucose metabolism in diabetes complications, as it mirrors glycosylation of proteins [260].

HbA1c an integrated measure of the level of glycemia, was positively associated with the prevalence of abnormal albumin excretion among diabetic patients. At high HbA1c values, which are indicative of high blood glucose concentrations, microalbuminuria is most likely caused by the deleterious effects of hyperglycemia on cell functions and extra cellular structures such as hypertrophy and basement membrane thickening [261], increased endothelial cell permeability to albumin [262], increased matrix protein synthesis [263] and increased production of
vasodilatory prostaglandins, which contribute to renal hyper perfusion, intraglomerular hypertension, and increased hyperfiltration [264].

The association of diabetic glomerulopathy with increased renal production of type IV collagen, a prominent constituent of the thickened basement membrane and expanded mesangium [265-267], has prompted measurement of the concentration of this extracellular matrix protein in biologic fluids in the hope that such measurements might serve as a useful indicator of early diabetic renal disease [268-270]. Collagen type IV (CIV) is uniquely present in basement membranes and represents their predominant structural element [271,272]. Metabolic alteration of collagen IV occurs in micro- or macrovascular basement membrane of diabetic patients. Immunostaining for the α-chains of type IV collagen has been used for the detection of GBM changes in several glomerular diseases, including thin GBM disease and IgA nephropathy [273-275].

To develop ground-breaking therapeutic options to prevent the development and progression of DN, a comprehensive understanding of the molecular mechanisms involved in the pathogenesis of this disease is mandatory. Therefore, the aim of this study was to examine the effects of NARN on the progression of DN in STZ-induced diabetic rats and discuss the underlying mechanism.

7.1 RESULTS

7.1.1 Anti-inflammatory effects of NARN on STZ-induced diabetic nephropathy

Table 7.1, figure 7.1 and 7.2 show the levels of NARN on serum immunoglobulin and renal tissues cytokines in STZ induced diabetic nephropathy. The increased levels of cytokines (TNF-α, IL-1, IL-6 and IL-8) were observed in STZ
induced diabetic nephropathy and its decrease with the concurrent treatment with NARN at a dose of 50 mg/kg b.wt/day for 15 days suggests that it has anti-inflammatory activity. Animals treated with AG decrease the levels renal tissues cytokines (group IV). Rats treated with NARN alone showed no significant differences in renal tissues cytokines levels as compared to control animals.

7.1.2 Effect of NARN treatment on the immunoglobulins (IgA, IgG) levels in diabetic nephropathy

Table 7.1 and figure 7.1 show the levels of NARN on serum immunoglobulin in STZ induced diabetic nephropathy. The levels of immunoglobulins (IgA, IgG) were significantly increased as compared to control animals (group I). Oral administration of NARN (50 mg/kg b.wt/day for 15 days) and AG (100 mg/kg b.wt/day for 15 days; i.p) animals revert back the status of IgA and IgG to near normal concentration. Animals treated with NARN alone showed no significant differences in IgA and IgG levels as compared to control animals.
**Table 7.1**: Effect of NARN on serum immunoglobulin and cytokines (renal tissues) in STZ induced diabetic nephropathy.

<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>IgG (mg/dl)</td>
<td>541.19±37.43a</td>
<td>747.83±40.15b</td>
<td>584.93±37.18a</td>
<td>575.86±31.37a</td>
<td>545.59±28.69a</td>
</tr>
<tr>
<td>IgA (mg/dl)</td>
<td>102.48±6.14a</td>
<td>365.22±21.87b</td>
<td>165.88±9.93c</td>
<td>139.04±8.33d</td>
<td>116.02±6.95a</td>
</tr>
<tr>
<td>TNF-α (pg/mg protein)</td>
<td>75.21±4.51a</td>
<td>172.25±10.32b</td>
<td>101.30±6.06c</td>
<td>90.02±5.39d</td>
<td>80.14±4.79a</td>
</tr>
<tr>
<td>IL-1(pg/mg protein)</td>
<td>22.96±1.37a</td>
<td>65.02±3.89b</td>
<td>32.88±1.97c</td>
<td>31.10±1.86c</td>
<td>25.99±1.56d</td>
</tr>
<tr>
<td>IL-6(pg/mg protein)</td>
<td>53.13±3.182a</td>
<td>159.58±9.56b</td>
<td>100.03±5.99c</td>
<td>85.67±5.13d</td>
<td>58.13±3.48a</td>
</tr>
<tr>
<td>IL-18 (pg/mg protein)</td>
<td>31.41±2.39a</td>
<td>40.13±3.06b</td>
<td>34.02±2.59a</td>
<td>33.06±2.51a</td>
<td>32.01±2.44a</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.
Figure 7.1: 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).
Figure 7.2: Effect of NARN on cytokines (renal tissues) 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).
7.1.3 Effect of NARN treatment on glucose and glycosylated haemoglobin (HbA1c) levels

The levels of glucose and HbA1c measured at the end of 15 days of different experimental groups are represented in table 7.2 and figure 7.3. A significant (p<0.05) increase in HbA1c (12.65±0.69) and glucose (452.08±34.42) levels were observed in the STZ induced DN group II animals, when compared with normal control animals. Treatment of NARN and AG in STZ diabetic rats from 2nd day to 15th day ameliorated the HbA1c and glucose levels significantly (p<0.05) as compared with group II animals, indicating NARN works as a better hypoglycemic agent. Furthermore, no significant differences were observed in NARN alone treated animals as compared to control animals.

7.1.4 Effect of NARN treatment on the collagen IV levels

Table 7.2 and 7.4 shows the status of collagen IV in serum of the control and experimental groups. The concentration of collagen IV was significantly increased in STZ induced diabetic nephropathy group II as compared to control animals. Oral administration of NARN (group III) and AG (group IV) significantly decreased the levels of collagen IV. No significant differences were observed in the levels of collagen IV in NARN (group V) alone treated animals as compared to control animals.
Table 7.2: Effects of NARN on serum glucose, HbA1c and collagen IV in STZ-induced diabetic nephropathy

<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>Glucose (mg/dL)</td>
<td>100.02±7.62&lt;sup&gt;a&lt;/sup&gt;</td>
<td>452.08±34.42&lt;sup&gt;b&lt;/sup&gt;</td>
<td>150.14±11.43&lt;sup&gt;c&lt;/sup&gt;</td>
<td>140.93±10.73&lt;sup&gt;c&lt;/sup&gt;</td>
<td>115.02±8.76&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>3.25±0.18&lt;sup&gt;a&lt;/sup&gt;</td>
<td>12.65±0.69&lt;sup&gt;b&lt;/sup&gt;</td>
<td>7.88±0.43&lt;sup&gt;c&lt;/sup&gt;</td>
<td>6.23±0.34&lt;sup&gt;d&lt;/sup&gt;</td>
<td>3.55±0.19&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Collagen IV (pg/ml)</td>
<td>80.03±4.79&lt;sup&gt;a&lt;/sup&gt;</td>
<td>155.48±9.31&lt;sup&gt;b&lt;/sup&gt;</td>
<td>98.14±5.88&lt;sup&gt;c&lt;/sup&gt;</td>
<td>85.14±5.09&lt;sup&gt;a&lt;/sup&gt;</td>
<td>82.15±4.92&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
Figure 7.3: Effect of NARN on serum glucose and HbA1c 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).
Figure 7.4: Effect of NARN on collagen IV in STZ-induced diabetic nephropathy.

The values that do not share a common superscript letter (a, b and c) between groups differ significantly at p<0.05 (Analysis of variance followed by DMRT; n=10).
7.2 DISCUSSION

Kidney is a vital organ that removes waste products of tissue metabolism through plasma intourine and maintains homeostasis of essential cellular biomolecules [276]. Defect in kidney leads to deleterious effect on normal physiology vice versa long term changes in normal physiology (chronic hyperglycemia) will also affect thenormal functioning of kidney and its structure. One such pathological change of kidney in diabetes is decrease drenal function that includes glomeruli [277].

DNis characterized by micro albuminuria, renal andglomerular hypertrophy, mesangial expansion with glomerular basement membrane thickening, arteriolar hyalinosis and global glomerular sclerosis, which ultimately cause the progression of proteinuria and renal failure [262,278]. Hyper glycemia is a necessary precondition for the development of the lesions [184,278], whereas systemic hypertension is an equally important aggravating factor of the disease [184, 279].

During DN, increase in type IV collagen and decrease in heparan sulfate (HS) are known to take place [280]. Type IV collagen is one of the first known constituents of the ECM in glomerular basement membrane that provides a scaffold for other ECM components by its network-like structure [281]. Much of the perm selectivity to circulating macromolecules is dependent upon this structure. GBM thickening and altered composition during DN leads to abnormal filtration [282].

Advanced glycation end products (AGEs) have been implicated in the pathogenesis of diabetic complications and the irreversible formation of AGEs promotes deposition of the ECM such as collagen in the glomeruli via RAGE and abnormal glomerular remodeling in the kidney [283, 284]. Inhibitors of AGES formation and cross-linking prevent the progression of DN [285,286].Cytokines are
small protein produced by numerous cells in the body that play a part in the process of cell signaling.

The production of macrophages depends on leukocytes being able to differentiate. The differentiation of monocytes into macrophages begins with the formation of pro-monocytes found within the bone marrow. Monocytes circulate in the blood until they migrate to tissues and inflammatory sites where they differentiate into macrophages [287]. Macrophages are mononuclear phagocytes, which function as scavengers that engulf bacteria, viruses, apoptotic cells, debris, and damaged cells. Cellular activation of macrophages begins once there is interaction with antigens, which causes alterations in morphology of macrophages increasing phagocytosis, chemotaxis, cytotoxicity and the production of inflammatory mediators [288]. There gulation of macrophages occurs through interactions with inflammatory mediators and cytokines. Cytokines comprise an extensive class of cell-derived proteins that protect a host from cellular injury, microbial invasion or agents that cause inflammation. The main cytokines secreted from macrophages include TNF-α, IL-1, IL-18, IL-12 and TGF-β [289, 290].

Accumulating evidences suggest that inflammation plays an important role in the development and progression of DN [60]. Moreover, circulating inflammatory markers and pro-inflammatory cytokines like TNF-α, IL-1, IL-6 and IL-18 are closely related with the risk of DN [60]. TNF-α, predominantly produced from activated macrophages and T cells, is a potent pro inflammatory cytokine and mediator of the inflammatory response. Although TNF-α is usually undetectable in healthy individuals; elevated serum and tissue levels of this molecule are found during periods
of inflammation and infection with its levels will vary according to the severity of the condition [291, 292].

Increased TNF-α level have been shown to alter metabolic pathways involved in lipid, cholesterol, triglyceride and glucose metabolism, contributing to the development of chronic diseases such as atherosclerosis and DN [293] of group II rats. TNF-α expression levels have been observed in the tubulointerstitium as well as in proximal tubular epithelial cells and elevated levels of TNF-α have been noted in STZ-induced DN animals (group II) to play a crucial role in renal injury by the reduction of blood flow and filtration rate and the alteration of the barrier function of capillary walls [294].

IL-1 was first implicated in the development of DN when glomerular basement proteins isolated from STZ-induced DN male rats (group II) had significantly greater macrophage, TNF-α, and IL-1 production compared to control rats [295]. Kidney tissues IL-6 levels are also significantly greater in diabetic nephropathy animals with overt proteinuria compared to normoalbuminuric and microalbuminuric animals [296, 297].

IL-18 also stimulates the production of other inflammatory cytokines including IL-1, TNF-α, and IL-6, upregulates ICAM-1 expression and induces endothelial cell apoptosis [245, 249]. This raises the possibility that an increase in IL-18 in DN animals (group II) may proceed the observed increase in IL-6. IL-18 is constitutively expressed in renal tubularepithelia and infiltrating monocytes, macrophages and proximal tubular cells have all been identified as potential sources of IL-18 production [245, 298].
In addition, macrophage accumulation and activation in the kidney contributed to albuminuria and renal fibrosis [299]. However, with recent advancement in understanding of DN, anti-inflammatory therapy may emerge as another standard treatment for patients with DN [300]. NARN ameliorates the altered circulatory inflammatory markers and pro-inflammatory cytokines (group III) indicates its anti-inflammatory effects. The altered serum immunoglobulins (IgA and IgG) may play an important role in the host defense mechanism in diabetic patients [301]. DN animals showed a marked alteration in serum IgA and IgG, which could be attributed to their increased vascular clearance and to the extent of nephropathy. Non enzymatic product also interact with specific receptors and binding proteins to influence the renal expression of growth factors and cytokines, implicated in the progression of diabetic renal disease [302-304].

The most important finding in our study was that the animals with DN showed higher levels of serum IgA in comparison to normal control animals. This finding is consistent with the data reported by Gonzales-Quinetela A et al[305]. STZ-induced diabetic rats have been described as a useful experimental model to study the preventive effect of DN because the selective destruction of pancreatic $\beta$-cells by STZ leads to the poor sensitivity of insulin for glucose uptake [306]. Increased blood glucose levels and AGEs formation are involved in the development of DN [283] in group II animals. Although the NARN or AG treated diabetic groups, these levels were significantly decreased. This effect on blood glucose control is reflected in the HbA1c levels compared with the untreated diabetic group II (table 7.2).

Erythrocytes are freely permeable to glucose. In cells glucose attaches to the free amino ends of hemoglobin molecules and lead to glycation of hemoglobin.
Glycosylated hemoglobin to be formed is directly proportional to the blood glucose concentration. The average erythrocyte life span is about 120 days, glycosylated hemoglobin levels give information on the mean average blood glucose levels over the past 2 to 3 months [307]. Two main fraction of glycosylated hemoglobin HbAI or HbA1c are commonly used in diabetes monitoring. The normal range for HbA1c 4-6 percent depending upon the assay [308].

Prolonged episodes of hyperglycemia lead to higher ratios of HbA1c to unmodified hemoglobin, which is produced in a non-enzymatic process when hemoglobin is exposed to glucose, and allows it to be used as a surrogate index of glycemic control [309]. A recent report from the World Health Organization defines HbA1c levels greater than 6.5% as a diagnosis for diabetes. However, since increased levels of HbA1c are only found after chronic exposure to increased glucose concentrations, early stage diabetes can be present with normal HbA1c ratios.

Striking consequential effects of prolong hyperglycemia are changes in structure and function of macromolecules [310, 311]. The architecture of the kidney changes (chapter VI) over time in response to chronic hyperglycemia and is characterized by cellular hypertrophy and increased matrix deposition [312]. Electron microscopy of renal biopsy sections shows nodular and diffuse lesions around the capillaries and towards the glomerular tuft (figure 6.14 and 6.15) [313].

In this investigation, NARN had a significant effect on limiting the increase in blood glucose levels which might be due to its hypoglycaemic effect [196]. Hyperglycemia results in increased glycosylation of a number of proteins, including haemoglobin and the observed reduction in the levels of HbA1c might be due to the free radical-quenching property of the drug since agents with antioxidant or free
radical-scavenging properties are capable of inhibiting oxidative reactions associated with protein glycation [314].

Excessive deposition of type IV collagen is an established feature associated with diabetic glomerulopathy [315]. The reaction between glucose and the lysine amino terminal of circulating and structural protein gives rise to glycosylation products by a non-enzymatic process [316]. Non-enzymatic glycosylation affects the GBM and other matrix components in the glomerulus. Excess GBM glycosylation, as seen in diabetes, may lead to an increase in the degree of cross linking of disulfide bridges between collagen components via increased oxidation of sulfhydryl groups. This process may induce molecular rearrangement and has been implicated in cataract formation in the lenses [317]. Similar cross-linking by disulfide bonds might affect the assembly and architecture of the GBM and mesangial matrix of group II DN rats. It has also been shown that AGEs are capable of extensive cross-linking throughout the collagen molecule, the cross-linking of collagen to lipoproteins as well as the thinking of GBM and glomerular trapping of IgG molecules [318].

In summary, we demonstrated that the administration of NARN effectively ameliorated alterations in early DN induced by STZ. The finding suggests that NARN may be useful in the prevention of hyperglycemia in early DN. However, the inhibitory effects of NARN on expressions of TNF-α and collagen IV and oxidative stress (chapter VIII) diabetic rat imply its potential efficacy in preventing the progression of DN.