Chapter 5

Effect of treatments on CD36 expression in preventing the progression of DNP
5.1 BACKGROUND

Though DNP is considered as a multifactorial disease, the formation of advanced glycation end products induced by hyperglycemia and dyslipidemia are considered to play a central role in mediating the progression of DNP (Rosario and Prabhakar, 2006). So, interest has been focused on therapeutic interventions targeting hyperglycemia and dyslipidemia for preventing the progression of DNP. In search of a novel drug for preventing the progression of DNP, aged garlic extract was identified.

Garlic is a culinary herb with remarkable medicinal properties and it has been used historically for the treatment of diseases associated with ageing. Garlic has been prepared and utilized in various forms such as garlic powder, garlic oil, aged garlic extract (GE) and aqueous garlic extract. Of all the aforementioned garlic preparations, GE is found to have the maximum beneficial effects (Morihara et al., 2001). GE has been proven to exhibit antioxidant properties both in vitro and in vivo (Moriguchi et al., 2001). The constituents of GE possess anti-thrombotic effect (Fukao et al., 2007) and it has been proven to protect against coronary heart diseases. Epidemiological studies have revealed that the consumption of garlic has decreased the incidence of certain forms of cancer including stomach, colon and laryngeal cancers (Fleischauer et al., 2001). GE has the ability to inhibit the oxidation of LDL in vitro, thus inhibiting lipoprotein modifications (Lau, 2006). Garlic has been found to reduce the plasma lipid and cholesterol level, and the lipid lowering effect is attributed to the decreased activities of lipogenic and cholesterogenic enzymes (Mahmoodi et al., 2006). The supplementation of GE has also shown hypoglycemic and hypotensive properties in animal models (Saravanan et al., 2009). Hence, this study was aimed to explore the anti-glycation and hypolipidemic property of aged garlic extract and demonstrate its renoprotective effect in experimentally induced diabetic rats.

CD36 has been proven to be associated with insulin resistance, hyperglycemia, and atherosclerosis (Handberg et al., 2008, Fernandez-Real et al., 2009). It has also been reported that CD36 uptakes advanced glycation end products and advanced oxidation protein products, which in turn mediates tubular epithelial cell apoptosis, thus
contributing for nephropathy (Susztak et al., 2005). In our previous study, we found that the CD36 m-RNA and protein expression in experimental rat kidney was increased with the progression of DNP. Further, the level of sCD36 in plasma and urine were also found to be increased in DNP rats and this was later confirmed in the human sample analyses. We found that there is increased level of soluble CD36 in plasma and urine of diabetic patients with microalbuminuria and macroalbuminuria. This showed the pathological importance of CD36 in mediating renal injury in diabetic condition. Since CD36 was found to have a strong association with the progression of DNP, this study was designed to analyze the effect of intervening substances that include insulin, aminoguanidine and aged garlic extract on CD36 m-RNA and protein expression and the level of soluble CD36 in plasma and urine.
5.2 MATERIALS AND METHODS

5.2.1 CHEMICALS AND REAGENTS

Kyolic® Aged garlic extract was procured from Wakunaga of America Co., Ltd., USA. Aminoguanidine and Streptozotocin (STZ) were purchased from Sigma Aldrich, India. The kits for albumin, urea, triglycerides, total cholesterol and HDL-Cholesterol were obtained from Span Diagnostics Ltd., Gujarat, India. The glycated hemoglobin kit was procured from Euro Diagnostics Ltd., Chennai. All the other reagents and chemicals were obtained from Sisco Research Laboratories Ltd., India.

5.2.2 EXPERIMENTAL DESIGN

Eight weeks old male albino Wistar rats weighing 200-250 g were used for the study. The rats were maintained under standard laboratory conditions (22 ± 3 °C, 12-h light/dark cycle) supplied with pelleted food and water ad libitum in VIT Animal house, Vellore. The animals were cared in accordance with the guidelines provided by the CPCSEA and the Institutional Animal Ethical Committee approved the entire study (Approval no.VIT/ IAEC /IV/ 031/ 2011).

5.2.3 INDUCTION OF DIABETES

Streptozotocin (STZ), at a dose of 45 mg/kg body weight dissolved in citrate buffer, was injected i.p. for inducing diabetes. The rats were fasted for 16 hours before the STZ injection, and 5% sucrose was supplemented for 48 hours post STZ injection in order to prevent the rats from fatal hypoglycemia. One week after STZ injection, the blood glucose level was analyzed using glucometer. The animals with a random blood glucose level of more than 300 mg/dl were considered diabetic and included in the study.

The rats were allocated into six groups with six rats in each group. The groups include control rats (Con), control rats supplemented with 500 mg/kg body weight of GE (Con + GE), STZ induced diabetic rats (Dia), diabetic rats treated with 4 units/day of insulin, diabetic rats administered with 1 g/L of aminoguanidine (Dia + AMG) dissolved in drinking water, diabetic rats supplemented orally with GE (Dia + GE) at a dose of 500
mg/kg body weight. Based on the findings of Omotoso et al., 500 mg/kg body weight/day of GE was considered as the optimum dosage and was used in our study (Omotoso et al., 2011). The treatment was started after two weeks of STZ injection, once the rats recovered from mild nephrotoxic effects of STZ and it was continued for twelve weeks.

5.2.4 BIOCHEMICAL ANALYSES

5.2.4.1 DETERMINATION OF GLYCATED HEMOGLOBIN CONTENT

Glycated hemoglobin level was analyzed by ion-exchange resin method as described in the product insert of Euro Diagnostics Ltd., Chennai. Briefly, 50 µl of blood was lysed using the lysing reagent and the hemolysate prepared was added to the ion exchange resin tube. The resin separator was inserted into the tube, such that the rubber sleeve is 1 cm above the resin suspension and the tubes were vortexed. The resin separator was pushed in completely to remove the supernatant and the absorbance was measured at 415 nm against distilled water to obtain ΔGHb. 20 µl of hemolysate was added to 5 ml of distilled water and the absorbance was measured at 415 nm to get ΔTotalHb. The percentage of glycated hemoglobin content was then calculated using the formula,

\[ \text{GHb\%} = \left( \frac{\Delta \text{GHb}}{\Delta \text{TotalHb}} \right) \times 4.61 \] (assay factor)

5.2.4.2 MEASUREMENT OF SERUM AND URINE ALBUMIN

Albumin content was quantified based on bromocresol green method using the kit procured from the Span Diagnostics Ltd., Gujarat, India. Briefly, 1 ml of reagent was added to 10 µl of sample and the absorbance was measured after 1 min incubation. Albumin present in the sample binds to the anionic dye bromocresol green, forming a green colored complex. The absorbance measured at 600 nm was compared with the standard and the albumin content was expressed in g/dl.

5.2.4.3 QUANTIFICATION OF SERUM AND URINE CREATININE

Creatinine content was analyzed based on Jaffe method followed by Farrell and Bailey, (1991). Briefly, 2 µl of sample (serum/urine) was added to 240 µl of working
reagent and the mixture was incubated at room temperature for 30 min. After incubation, the absorbance was read at 505 nm. 20 µl of 30% acetic acid was added to the wells and incubated further for 10 min. The absorbance was again read at 505 nm and the difference in absorbance was calculated. The concentration of creatinine was then estimated by comparing with the standard.

5.2.4.4 MEASUREMENT OF BLOOD AND URINE UREA NITROGEN

Urea nitrogen content was analyzed using the kit procured from the Span Diagnostics Ltd., Gujarat, India. Briefly, 10 µl of diluted urine (1: 20 v/v) or serum was added to the reagents and incubated in boiling water bath for 10 min. Urea present in the sample reacted with diacetylmonoxime in the presence of thiosemicarbazide to form a purple colored complex, which was measured at 525 nm. The absorbance was compared with that of the standard and the urea nitrogen content was calculated.

5.2.4.5 QUANTIFICATION OF SERUM LIPID PROFILE

The serum lipid parameters such as total cholesterol, triglycerides and high density lipoprotein (HDL) cholesterol were estimated by the enzymatic CHOD-PAP method, GPO-PAP method, and PEG-CHOD-PAP method respectively using the kit procured from the Span Diagnostics Ltd., Gujarat, India. The LDL cholesterol and the atherogenic index (AI) were estimated based on the Friedewald equation (Friedewald et al., 1972).

\[
\text{LDL- cholesterol} = \text{Total cholesterol} - (\text{Triglycerides}/5 - \text{HDL cholesterol})
\]

\[
\text{AI} = (\text{Total cholesterol} - \text{HDL cholesterol})/ \text{HDL cholesterol}
\]

5.2.5 HISTOPATHOLOGICAL EXAMINATION

Two rats from each group were sacrificed under mild anesthesia at the end of 12 weeks of treatment. Kidneys were excised carefully without any damage, washed in PBS and stored in 10% neutral buffered formalin. The kidneys were then processed and embedded in paraffin. 4 micron sections were cut and stained with H&E and Periodic Acid Schiff base for histological examination. The sections were then observed for the
degree of tubular and glomerular damage. Glomerular damage index (GDI) was calculated and scored from 0 to 4 on the basis of the degree of glomerulosclerosis, mesangiolysis and mesangial expansion. 80 to 100 glomeruli from renal cortex were observed for each sections and GDI was obtained by averaging the scores from the counted glomeruli (Raji et al., 1984).

5.2.6 ANALYSIS OF CD36 m-RNA EXPRESSION

The m-RNA expression of CD36 was observed in Real time PCR as described in chapter 2

5.2.7 IMMUNOHISTOCHEMICAL ANALYSIS OF CD36 EXPRESSION

The immunohistochemical observation of AGE and CD36 in kidney was performed as described in chapter 2.

5.2.8 ANALYSIS OF AGE AND CD36 PROTEIN EXPRESSION IN KIDNEY

The AGE and CD36 protein expression analysis was done using western blot and the relative expression was analyzed using densitometry, as described in chapter 2.

5.2.9 ANALYSIS OF THE LEVEL OF CD36 IN PLASMA AND URINE

The level of soluble CD36 in plasma and urine was analyzed using Sandwich ELISA as described in chapter 3.

5.2.10 STATISTICAL ANALYSIS

The values are represented as mean ± S.D (n=6) and the data were analyzed on Graph Pad *Prism* 5.01 software. One-way ANOVA followed by Dunnett’s test was performed to compare the diseased and the treated groups. The statistical difference between the normal and diseased was analyzed by Un-paired *t*-test. The results were considered statistically significant, if *p* ≤ 0.05.
5.3 RESULTS

5.3.1 EFFECT OF AGED GARLIC EXTRACT ON BODY WEIGHT AND URINE VOLUME

The diabetic rats showed a significant decrease (p< 0.001) in the body weight throughout the study period, compared to control rats (Table 5.1). Though there was a mild improvement in the body weight of the animals that were treated with insulin and aminoguanidine, supplementation of aged garlic extract decreased the body weight of the animals. Our results coincided with the finding that the supplementation of GE resulted in weight loss in diabetic rats (Morbidoni et al., 2001).

In general, the volume of urine excreted by a normal adult rat remains the same. Because of the osmotic imbalance between the body fluids and cellular contents, induced by hyperglycemia, the diabetic rats showed a predominant increase in the urine volume (p< 0.001). In diabetic rats treatment with insulin, aminoguanidine and GE, the urine volume was significantly decreased (p< 0.001) and at the end of the study, it was very close to normal (Table 5.1).

5.3.2 EFFECT OF AGED GARLIC EXTRACT ON BLOOD GLUCOSE AND GLYCATED HEMOGLOBIN CONTENT

The blood glucose level of the diabetic rats was significantly high (~ 600 mg/dl) throughout the study period (p< 0.001). In diabetic rats that were treated with insulin and aminoguanidine, there was a significant decrease in the blood glucose level. But the GE supplementation elicited no significant difference in the blood glucose level (Table 5.1). Our results are highly contradictory to the results obtained by Saravanan et al., 2009, who demonstrated the anti-diabetic effect of S-allyl cysteine, a major constituent of GE. Though GE supplementation did not show a significant effect on the blood glucose level, its effect on the glycated hemoglobin content was significant (p< 0.05) and was comparable with that of the insulin and aminoguanidine treated animals. This may be attributed to the anti-glycation activity of GE (Yeh and Liu, 2001).
<table>
<thead>
<tr>
<th>Parameters</th>
<th>Groups</th>
<th>Con</th>
<th>Dia</th>
<th>Dia + Ins</th>
<th>Dia + AMG</th>
<th>Dia + GE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td></td>
<td>295 ± 3</td>
<td>151 ± 2***</td>
<td>240 ± 7</td>
<td>186 ± 3*</td>
<td>153 ± 1.4</td>
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<tr>
<td>Urine Volume (ml)</td>
<td></td>
<td>3.8 ± 0.4</td>
<td>24.5 ± 1</td>
<td>8.3 ± 0.4***</td>
<td>9 ± 1.4</td>
<td>8.6 ± 0.6</td>
</tr>
<tr>
<td>Blood Glucose (mg/dl)</td>
<td></td>
<td>100 ± 2</td>
<td>582 ± 12***</td>
<td>212 ± 17***</td>
<td>433 ± 6***</td>
<td>552 ± 7</td>
</tr>
<tr>
<td>HbA1C (%)</td>
<td></td>
<td>6 ± 0.7</td>
<td>9.8 ± 0.2#</td>
<td>6.9 ± 0.5***</td>
<td>7.6 ± 0.7*</td>
<td>8.3 ± 0.8*</td>
</tr>
<tr>
<td>Albumin (mg/dl)</td>
<td></td>
<td>4.1 ± 0.1</td>
<td>3.2 ± 0.2#</td>
<td>3.4 ± 0.1</td>
<td>3.4 ± 0.1</td>
<td>3.4 ± 0.1</td>
</tr>
<tr>
<td>Creatinine (mg/dl)</td>
<td></td>
<td>0.9 ± 0.1</td>
<td>1.74 ± 0.3##</td>
<td>1.2 ± 0.2</td>
<td>1.49 ± 0.03**</td>
<td>1.57 ± 0.12**</td>
</tr>
<tr>
<td>Urea Nitrogen (mg/dl)</td>
<td></td>
<td>15 ± 0.5</td>
<td>59 ± 3.5##</td>
<td>19 ± 2.9***</td>
<td>21 ± 3.8**</td>
<td>29 ± 3.7**</td>
</tr>
</tbody>
</table>

Table 5.1: Effect of aged garlic extract on metabolic parameters and serum biochemistry

Metabolic parameters and serum biochemistry of the animals from the five groups; Con, Con + GE, Dia, Dia + AMG, Dia + GE at the end of 12 weeks. Values represent the mean ± standard deviation of the samples (n=6). Significant difference between Con and Dia groups: # p< 0.05; ## p< 0.01; ### p< 0.001. Significant difference for Dia Vs Dia + Ins, Dia Vs Dia + AMG, Dia Vs Dia + GE groups: * p< 0.05; ** p< 0.01; *** p< 0.001.
5.3.3 EFFECT OF AGED GARLIC EXTRACT ON THE BIOMARKER OF DNP

Diabetic nephropathy is being diagnosed clinically by an increase in the level of albumin in urine (albuminuria), accompanied by its decrease in serum. In our study, significant increase in the albumin content was observed in diabetic rat urine (p< 0.001), with a significant decrease in the level of albumin in serum (p< 0.001) (Table 5.1). This showed that the rats that were induced with diabetes have progressed to DNP. In diabetic rats supplemented with GE, there was a significant decrease in the albumin level in urine (p< 0.01), which was similar to that observed in insulin and aminoguanidine administered rats (Figure 5.1). At the end of the study, the albumin level of the diabetic rats supplemented with GE was brought back to normal, which confirmed that GE has the ability to attenuate DNP.

5.3.4 IMPROVEMENT OF CREATININE CONTENT AFTER SUPPLEMENTATION OF AGED GARLIC EXTRACT

The kidney damage, in general, is reflected by a significant decrease in the creatinine content in urine and DNP is no exception. The diabetic rats showed a significant decrease in the level of creatinine in urine (p< 0.001) with a significant increase in serum (p< 0.001), which confirmed that the animals have encountered kidney damage (Figure 5.2). However, in diabetic rats supplemented with GE, the creatinine content increased significantly in urine (p< 0.05) with a significant decrease in serum (p< 0.01). This led us to confirm that the supplementation of GE could improve the kidney damage in diabetic rats.
Figure 5.1: Effect of treatment on microalbuminuria

Graph showing the urine albumin level (mg per 12 h) of the animals from the groups; Con, Con + GE, Dia, Dia + AMG, and Dia + GE. Values are given as mean ± standard deviation (n=6). Significant difference between Con and Dia groups: # p˂ 0.05; ## p˂ 0.01; ### p˂ 0.001. Significant difference between Dia and Dia + AMG, Dia and Dia + GE groups: * p< 0.05; ** p< 0.01; *** p< 0.001.
Figure 5.2: Effect of treatment on creatinine content in urine

Graph showing the urinary creatinine level (mg/dl) of the animals from the groups; Con, Con + GE, Dia, Dia + AMG, Dia + GE. Values are given as mean ± standard deviation (n=6). Significant difference between Con and Dia groups: # p< 0.05; ## p< 0.01; ### p< 0.001. Significant difference between Dia and Dia + AMG, Dia and Dia + GE groups: * p< 0.05; ** p< 0.01; *** p< 0.001.
Figure 5.3: Effect of treatment on urea nitrogen content in urine

Graph showing the level of urine urea nitrogen (gram per litre) of the animals from the groups; Con, Con + GE, Dia, Dia + AMG, Dia + GE. Values are given as mean ± standard deviation (n=6). Significant difference between Con and Dia groups: # p< 0.05; ## p< 0.01; ### p< 0.001. Significant difference between Dia and Dia + AMG, Dia and Dia + GE groups: * p< 0.05; ** p< 0.01; *** p< 0.001.
5.3.5 IMPROVEMENT OF UREA NITROGEN CONTENT AFTER AGED GARLIC EXTRACT SUPPLEMENTATION

In diabetic patients, impaired glucose metabolism enhances the protein catabolism, increasing the level of urea in blood. The blood urea nitrogen level is further enhanced by the kidney damage induced by hyperglycemia. Thus, in DNP there was an increase in the level of blood urea nitrogen content with a decrease in its level in urine. The diabetic rats showed a significant increase (p< 0.001) in the blood urea nitrogen content and a significant decrease (p< 0.001) in the urine urea nitrogen content (Figure 5.3), which confirmed that the diabetic rats have encountered kidney damage. However, diabetic rats supplemented with GE showed a significant decrease (p< 0.01) in the blood urea nitrogen content (Table 5.1) with a significant increase (p< 0.01) in the urine urea nitrogen content, which was similar to that observed in insulin and aminoguanidine treated rats. This proved that GE could be used as a nephroprotectant in diabetic animals.

5.3.6 EFFECT OF AGED GARLIC EXTRACT ON SERUM LIPID PROFILE

In diabetic rats, significant increase in the level of triglycerides (p< 0.01), total cholesterol (p< 0.01), and LDL-cholesterol (p< 0.01) was observed with a significant decrease in the HDL-cholesterol level (p< 0.05). However, the diabetic rats that were supplemented with GE showed a significant decrease in the level of triglycerides (p< 0.05), total cholesterol (p< 0.01), and LDL-cholesterol (p< 0.01) with a significant increase in the HDL-cholesterol level (p< 0.05) (Table 5.2). At the end of the study, the serum lipid profile of the diabetic animals supplemented with GE was highly comparable with that of the insulin and aminoguanidine treated diabetic rats.
<table>
<thead>
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<th>Serum Parameters</th>
<th>Groups</th>
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<tbody>
<tr>
<td></td>
<td>Con</td>
</tr>
<tr>
<td>Triglycerides (mg/dl)</td>
<td>50 ± 5</td>
</tr>
<tr>
<td>Total Cholesterol (mg/dl)</td>
<td>103 ± 3.8</td>
</tr>
<tr>
<td>LDL-Cholesterol (mg/dl)</td>
<td>42 ± 0.7</td>
</tr>
<tr>
<td>HDL-Cholesterol (mg/dl)</td>
<td>50 ± 0.1</td>
</tr>
<tr>
<td>Atherogenic Index (AI)</td>
<td>1.0 ± 0.1</td>
</tr>
<tr>
<td>LDL/HDL ratio</td>
<td>0.82 ± 0.1</td>
</tr>
</tbody>
</table>

**Table 5.2: Effect of treatment on serum lipid profile**

Serum lipid profile of the animals from the five groups; Con, Con + GE, Dia, Dia + AMG, Dia + GE at the end of 12 weeks of treatment. Values represent the mean ± standard deviation of the samples (n=6). Significant difference between Con and Dia groups: # p< 0.05; ## p< 0.01; ### p< 0.001. Significant difference for Dia Vs Dia + Ins, Dia Vs Dia + AMG, Dia Vs Dia + GE groups: * p< 0.05; ** p< 0.01; *** p< 0.001.
5.3.7 EFFECT OF AGED GARLIC EXTRACT ON KIDNEY HISTOLOGY

The diabetic rats showed tubular changes such as glycosuria and proteinuria with mild mesangial expansion and proliferation in the glomeruli. Prominent nodular glomerulosclerosis with glomerular basement membrane thickening was also observed (Figure 5.5). Glomerulosclerosis was observed in more than 30% of the glomerulus in diabetic rats. But in diabetic rats treated with aminoguanidine and AGE, less than 10% of the glomerulus has encountered sclerosis (Figure 5.6). Further, minimal changes such as glycosuria and proteinuria were observed in the diabetic animals that were supplemented with GE, aminoguanidine and insulin (Figure 5.7), which proved that GE has the potential to ameliorate diabetic nephropathy.

5.3.8 EFFECT OF TREATMENT ON CD36 m-RNA EXPRESSION IN KIDNEY

In diabetic rats, there was a significant increase (p< 0.01) in the m-RNA expression of CD36 compared to the control rats (Figure 5.8). But in diabetic rats treated with insulin, there was significant decrease (p< 0.001) in the m-RNA expression of CD36. This showed the association of CD36 with blood glucose level. But in diabetic rats administered with aminoguanidine and GE, there was no change in the m-RNA expression of CD36 compared to diabetic rats.

5.3.9 EFFECT OF TREATMENT ON CD36 PROTEIN EXPRESSION IN KIDNEY

In the immunohistochemical analysis, very mild expression of CD36 was observed in the glomerular endocytes of control rats. The supplementation of garlic extract in control rats did not show any change in the expression of CD36. In diabetic rats, there was a significant increase in the distribution (> 80%) and intensity of expression of CD36 (Figure 5.9). In particular, there was a significant increase in CD36 expression in peritubular capillaries and in tubules. However, in diabetic rats treated with insulin, aminoguanidine and GE, the increase in CD36 expression was not so significant.

In the western blot, it was observed that there was significant increase (p< 0.001) in the expression of both AGE and CD36 in diabetic rats compared to control rats (Figure 5.10). The results were in accordance with the findings of Susztak et al., (2005) and were
similar to that we observed in our previous study. From the densitometric analysis, it was
found that there was significant decrease (p< 0.001) in AGE expression in diabetic rats
supplemented with insulin, aminoguanidine and aged garlic extract. Though the
administration of aminoguanidine showed a mild decrease (p< 0.05) in the expression of
CD36, insulin and aged garlic extract showed a significant decrease (p< 0.01) in CD36
expression at the end of three months of treatment (Figure 5.11).

5.3.10 EFFECT OF TREATMENT ON THE LEVEL OF SOLUBLE CD36 IN
PLASMA AND URINE

In diabetic rats, significant increase (p< 0.05) in the level of soluble CD36 was
observed in the plasma. In the treated groups, the level of soluble CD36 in plasma was
found to be similar to that observed in diabetic rats. Since the level of soluble CD36 in
plasma was found to be increased in the diabetic condition, it was speculated that the
CD36 level would decrease after treatment. But it was found that treatments had no effect
on the level of soluble CD36 in plasma (Figure 5.12). Since the rationale behind the
solubilisation of CD36 was not yet known, the reason for the increased level of CD36 in
plasma even after treatment could not be elucidated. The level of CD36 in urine was also
found to be increased significantly (p< 0.01) in diabetic rats. But in diabetic rats treated
with insulin, aminoguanidine and GE, the level of soluble CD36 decreased significantly
(p< 0.05) compared to diabetic rats (Figure 5.13). This implied that whenever the kidney
damage is improved in diabetic rats, the release of CD36 in urine is also decreased.
Figure 5.4: Kidney histology of control rats

Figure showing the images of kidney histology from control and rats supplemented with garlic extract. 4(a): control rats showing normal renal parenchyma, H&E, x 400; 4(b) (insert): control rats showing normal renal parenchyma, PAS, x 400; 4(c): rats supplemented with garlic extract showing normal glomerulus, tubules and blood vessels, H&E, x 400; 4(d) (insert): control rats supplemented with garlic extract showing normal glomerulus, tubules and blood vessels, PAS, x 400.
Figure 5.5: Kidney histology of diabetic rats

Figure showing the kidney histological images of diabetic rats. 5(a, b): The section shows mesangial expansion and nodular glomerulosclerosis with increase in glomerular capillary membrane thickening, H&E, PAS respectively, x 400.

Figure 5.6: Morphometric semiquantitative analysis of glomerular damage index

Glomerular damage index (GDI) was calculated on PAS stained sections with a scoring system of 0 to 4. 0: No lesions; 1+: sclerosis of < 25% of glomerulus; 2+: sclerosis of 25-50% of glomerulus; 3+: sclerosis of 50-75% of glomerulus; 4+: sclerosis of > 75% of glomerulus.
Figure 5.7: Kidney histology of diabetic rats treated with insulin, aminoguanidine and aged garlic extract

Figure showing the images of kidney histology from diabetic rats supplemented with aminoguanidine (a, b) and garlic extract (c, d). 6(a): section showing mild mesangial proliferation with normal glomerular capillary basement membrane thickening. Tubules and interstitium are within normal limits, H&E, x 400. 6(b) (insert): section showing mild mesangial proliferation with no mesangial nodules. Tubules and interstitium are within normal limits, PAS, x 400. 6(c, d (insert)): section showing glomerulus with normal cellularity and membrane thickness. Tubules, interstitium and blood vessels are within normal limits, H&E, PAS respectively, x 400.
Figure 5.8: Effect of treatment on CD36 m-RNA expression

Figure showing the effect of treatment on CD36 m-RNA expression. Values represent the mean ± standard deviation of the samples (n=6). *** Con Vs Dia sample, **** Dia Vs Dia + Ins, Dia + AMG, Dia + GE (***, ** p< 0.001; ***, ** p< 0.01; *, p< 0.05).
Figure 5.9: Effect of treatment on CD36 protein expression in kidney

Figure showing the immunohistochemical images of CD36 protein in all the six groups at the end of the study. Diabetic rats showed a significant increase in the expression of CD36. But in the diabetic rats treated with insulin, aminoguanidine and aged garlic extract, the increase was not that significant compared to the diabetic rats.
Figure 5.10: Western blot for AGE and CD36 in treated diabetic rat kidney

This figure shows the western blot images of AGE, CD36 and β-actin along with the densitometry for AGE and CD36 expression in relation to β-actin expression. Con Vs Dia sample, *****, Dia Vs Dia + Ins, Dia + AMG, Dia + GE (###,##,##,##,## p< 0.001; ##,**,## p< 0.01; *,## p< 0.05).

Figure 5.11: Densitometry for AGE and CD36 expression relative to β-actin

This figure shows the western blot images of AGE, CD36 and β-actin along with the densitometry for AGE and CD36 expression in relation to β-actin expression. Con Vs Dia sample, *****, Dia Vs Dia + Ins, Dia + AMG, Dia + GE (###,##,##,##,## p< 0.001; ##,**,## p< 0.01; *,## p< 0.05).
Figure 5.12: Effect of treatment on the level of soluble CD36 in plasma

Figure showing the effect of treatment on the level of soluble CD36 in plasma. Values represent the mean ± standard deviation of the samples (n=6). ###,##,# Con Vs Dia, ****** Dia Vs Dia + Ins, Dia + AMG, Dia + GE (###,*** p< 0.001; ##,** p< 0.01; * p< 0.05).
Figure 5.13: Effect of treatment on the level of soluble CD36 in urine

Figure showing the effect of treatment on the level of soluble CD36 in urine. Values represent the mean ± standard deviation of the samples (n=6). ***,##,# Con Vs Dia sample, 
*****,*** Dia Vs Dia + Ins, Dia + AMG, Dia + GE (***,*** p< 0.001; ###,** p< 0.01; #* p< 0.05) .
5.4 DISCUSSION

This study explored the effect of Kyolic® Aged garlic extract on DNP induced Wistar rats. The formation of AGE induced by hyperglycemia and hyperlipidemia are considered to play a major role in causing DNP. So any therapeutic intervention targeting both hyperglycemia and dyslipidemia would be considered a better treatment for DNP. Since GE was found to have hypolipidemic and hypoglycemic properties, this study has exploited the potential of GE in attenuating DNP.

Various pharmacological effects of GE have been identified that include its antioxidant (Moriguchi et al., 2001), anti-cancer (Yeh and Liu, 2001), anti-thrombotic (Fukao et al., 2007), hypolipidemic, hypocholesterolemic (Wang et al., 2012) and hypoglycemic activities (Saravanan et al., 2009). Since GE is composed of a mixture of phytochemicals, it is not surprising that it has tremendous biological properties. Though the active principle responsible for these biological activities is unknown, several studies have reported that these activities are pertained to S-Allyl cysteine, a major constituent of GE (Saravanan et al., 2009). Apart from S-Allyl cysteine, the other beneficial constituents of GE include alliin, γ-Glutamyl cysteine and S-Allyl mercaptocysteine, which are water soluble (Wang et al., 2012). The most common side effect associated with GE supplementation is weight loss (Morbidoni et al., 2001), which was observed in our study.

S-allyl cysteine (SAC) which is a major component of GE has been proven to be an effective anti-diabetic agent (Morbidoni et al., 2001). However, in our study, we did not observe any hypoglycemic effect for GE (Table 5.1). Though, there is no hypoglycemic effect in GE, its anti-glycation effect is noteworthy. At the end of 12 weeks, the glycated hemoglobin content of the diabetic rats that were supplemented with GE decreased significantly compared to that of the diabetic rats (Table 5.1). We presume that the supplementation of GE would have prevented the Amadori product formation, by acting as a cross-link inhibitor similar to aminoguanidine.
The supplementation of GE in diabetic rats has significantly reduced the level of triglycerides ($p<0.05$), total cholesterol ($p<0.01$), and LDL-cholesterol ($p<0.01$) with a small increase ($p<0.05$) in the HDL-cholesterol level (Table 5.2). This confirmed the hypolipidemic activity of GE, which was supported by several other independent investigations (Stevinson et al., 2000; Natural Standard Research Collaboration, 2010). The lipid lowering effect of GE may be pertained to di-allyl, di-sulphide, tri-sulphide compounds of garlic which have the ability to inhibit the activity of HMG-CoA reductase (Liu and Yeh, 2000). It has also been reported that the organosulphur compounds present in garlic have the ability to reduce the lipoprotein modification in vitro and in vivo (Wang et al., 2012). Since TGF-β induced by lipoprotein modification also plays a major role in the pathogenesis of DNP (Nakhjavani et al., 2010), inhibition of lipoprotein modification might also be significant along with its hypolipidemic effect in attenuating DNP.

In diabetic patients, when there is any damage to the glomerulus or the tubules, the proteins that have to be reabsorbed into the blood leak out in urine. Since albumin is the most abundant protein present in the blood, it is released easily in the urine and is used as a forerunner for DNP (Caramori et al., 2000). Further, kidney damage is reflected by an increase in the level of metabolic wastes such as creatinine and urea in the blood. In our study, the biochemical analyses showed evidence for albuminuria and kidney damage. This confirmed that the diabetic rats have progressed to diabetic nephropathy. However, the diabetic rats that were treated with GE showed a significant decrease ($p<0.01$) in albuminuria (Figure 5.1) with a significant change in urinary urea ($p<0.01$) and creatinine content ($p<0.05$) (Figure 5.2, 5.3). This proved that the GE supplementation has attenuated kidney damage in diabetic rats, thus ameliorating DNP.

In concordance with the results obtained in biochemical analyses, the kidney histological analysis of diabetic rats supplemented with GE showed mild mesangial expansion with no change in the glomerular basement membrane (Figure 5.7). The extent of glomerulosclerosis was also decreased significantly after GE supplementation, which is reflected by a significant decrease in the glomerular damage index (Figure 5.6). Further, the tubules, interstitium and the blood vessels were within normal limits, which revealed the efficacy of GE in treating DNP. Several investigators have proven the
renoprotective effect of GE on 5/6 nephrectomised rat model (Cruz et al., 2007; Bautista-Garcia et al., 2006) and nephrotoxic rat models (Maldonado et al., 2003; Wongmekiat et al., 2005). The renoprotective effect in these models was attributed to the anti-oxidant activity of GE. So it was speculated that the renoprotective effect of GE might be due to its antioxidant property. However, it was found that, apart from antioxidant effect, antglycation, hypolipidemic activity of GE also contribute for the ameliorative effect in DNP.

Once the renoprotective effect of aged garlic extract was established (Shiju et al., 2013), its effect on CD36 expression was analyzed along with insulin and aminoguanidine. In diabetic rat kidney, significant increase in CD36 m-RNA expression was observed compared to control rats (Figure 5.8). In the immunoblot, the increased m-RNA expression of CD36 was further substantiated by a significant increase in CD36 protein expression in diabetic rat kidney homogenate (Figure 5.10). Further, significant increase in the distribution of CD36 was also observed in kidney using immunohistochemical analysis (Figure 5.9). The results were very similar to that we observed in our previous study and were in accordance with the findings of Susztak et al. (2005). This confirms the pathological importance of CD36 in mediating the progression of DNP. In diabetic rats treated with insulin, there was a significant decrease (p< 0.001) in the m-RNA expression of CD36 (Figure 5.8) along with a significant decrease (p< 0.01) in CD36 protein expression in kidney homogenate (Figure 5.11). Since insulin is a potent anti-diabetic agent, there is very less chance for the formation of advanced glycation end products, thus decreasing the expression of CD36. In diabetic rats supplemented with aminoguanidine and GE, though there was a significant decrease (p< 0.05) in the CD36 protein expression in kidney, there was no change in the CD36 m-RNA expression. Supporting this finding, the tubular and the peritubular expression of CD36 were also decreased significantly in diabetic rats supplemented with GE, reflecting the renoprotective effect of GE.

In diabetic rats, significant increase (p< 0.05) in the level of soluble CD36 was observed in plasma (Figure 5.12). Further, the kidney damage in diabetic rats was corroborated by significant increase (p< 0.01) in the level of CD36 in urine. In our study,
aged garlic extract did not show a potential anti-hyperglycemic effect. So the renoprotective effect imparted in diabetic rats might be attributed to its anti-oxidant and hypolipidemic effects. Since the blood glucose level was not decreased significantly after supplementation of GE, there was no change in the level of soluble CD36 in plasma. Even in diabetic rat treated with insulin, though there was significant decrease in the blood glucose level, there was no change in the level of soluble CD36 in plasma. This led to the speculation that apart from hyperglycemic condition, there are other mechanisms that contribute for the increased level of CD36 in plasma. In diabetic condition, increased monocyte CD36 expression has been reported along with a strong association to IL-6. The low grade inflammatory state in the hyperglycemic condition was thought to contribute for the increased level of soluble CD36 in plasma. In diabetic rats administered with insulin, aminoguanidine and aged garlic extract, there was a significant decrease (p<0.01) in the level of CD36 in urine (Figure 5.13). This showed that whenever the kidney damage in diabetic rats was ameliorated, the level of CD36 in urine decreased significantly.
5.5 CONCLUSION

From the results, it was found that the supplementation of aged garlic extract has the ability to ameliorate kidney damage in diabetic rats, thus preventing the progression of DNP. The protective effect of GE on DNP may be attributed to its anti-glycation and hypolipidemic properties. The compound responsible for this attenuating effect might be S-allyl cysteine, which is a major constituent of GE. However, further research is required to find the active principle responsible for the attenuating effect of GE on DNP. Significant increase in the m-RNA and protein expression of CD36 in kidney with a significant increase in the level of soluble CD36 in plasma and urine, confirmed the pathological importance of CD36 in the progression of DNP. But when the kidney damage in diabetic rats was ameliorated by administering treatment in the form of insulin, aminoguanidine or aged garlic extract, there is significant decrease in the tubular expression of CD36 along with significant decrease in the level of CD36 in urine.