CHAPTER 4

4.1. INTRODUCTION

Non-enzymatic glycation of proteins is a post-translational modification process (Gillery and Jenison, 2014), leading to the formation of fructosamine (Lapolla et al., 2005) and AGEs (Goldin et al., 2006; Ahmad et al., 2013). It has also been reported that there is the generation of oxygen free radicals in the formation of early and advanced glycation end-products (Azevedo et al., 1988). The reaction proceeds by nucleophilic addition reaction followed by dehydration, cyclization and rearrangement to form AGEs. The role of glucose in the glycation of proteins has been widely studied however the role of other reducing monosaccharides such as D-ribose in glycation and their resulting effects on animal model has received much less attention. AGEs are heterogeneous class of compounds containing a mixture of cyclic and open chain structures out of which large numbers of AGEs are not determined till date. However, few AGEs which are known like pentosidine, carboxymethyl-lysine (CML), carboxyethyl-lysine (CEL), fructosyl-lysine (FL), which plays an important role in atherosclerosis and nephropathy. The lipoprotein glycation process occurs both on the apoprotein B (apoB) and on phospholipid components of LDL. It leads both functional alterations in LDL clearance and increased susceptibility to oxidative modifications (Mironova et al., 1997). In addition, oxidative stress causing oxidation of LDL stimulates the synthesis of nuclear polymers of ADP-ribose, which in turn can liberate monomeric ADP-ribose. The bioavailability of D-ribose makes this carbonyl species quite reactive and damaging, therefore having direct implication in diabetes.

There are ample evidences which indicate the role of lipoprotein glycation in the inflammatory reactions in the vessel wall (Akanji et al., 2002, Basta et al., 2004). Various oxidative stresses of different proteins may be involved in proinflammatory immune mechanisms. These may involve innate
immune signals, Toll-like receptors (Lundberg and Hansson, 2010) and adaptive immunity signals which are both cell-mediated (Andersson et al., 2010) and antibody mediated (Lopes-Virella and Virella, 2010). There are ample of evidences supporting the pathogenic role of humoral response to modified lipoproteins (Saad et al., 2006; Lopes-Virella et al., 2007; Yishak et al., 2006; Kummu et al., 2013). This is mainly due to the fact that modified LDL and its corresponding antibodies form modified LDL immune complexes (mLDL-IC), which are able to activate phagocytic cells through engagement of Fc receptors (Kummu et al., 2013). Engagement of Fc receptors by mLDL-IC is particularly significant because it delivers stronger activating signals to phagocytic cells than the engagement of scavenger receptors by modified LDL (Saad et al., 2006). Native LDL was found to be moderately immunogenic in experimental animals, while, modified form of LDL was found to be highly immunogenic (Steinberg, 2009).

This chapter was aimed to see the immunogenicity of native and glycated LDL. The cross reactivity experiments were performed to check the specificity of the raised antibodies using various glycated and non-glycated inhibitors including poly amino acids. Moreover, the immuno-histochemistry was performed to see the immune complex deposition in the kidney of immunized female rabbits.

4.2. MATERIAL AND METHODS

4.2.1. Ethics statement

The immunization work was approved by Institutional Animal Ethical Committee of Integral University, Lucknow, India by approval No: IU/ Biotech/ Project/ CPCSEA/ 12/ 20. The animals were treated with utmost care and treatments were done with minimum pain. The in vivo experiments on rabbits were performed according to the ARRIVE guidelines. After the end of
experiment the rabbits were sacrificed by the over dosage of thiopentone sodium anaesthesia by intra venous mode of injection (≥150 mg/kg).

4.2.2. Chemicals

D-ribose, Tris-HCl, EDTA, phosphotungstic acid (PTA), thiobarbituric acid (TBA), Protein A-Agarose column were obtained from Sigma, St. Louis, MO. Polystyrene plates obtained from Nunc (Roskilde, Denmark), HSA, Hb, IgG, Poly-L-lysine, Poly-L-arginine, Poly-L-histidine were obtained from MP Biomedicals and LDL, Anti rabbit IgG, p-nitrophenyl phosphate (pNPP) were purchased from Calbiochem. All other chemicals used in this study were of highest analytical grade available in the country.

4.2.3. Glycation of LDL, various proteins and amino acids

LDL was modified by using different concentrations of D-ribose (Chapter 3 Page no. 47; Ahmad et al., 2013). LDL (62.5µg/ml) was thoroughly mixed with 80 mM of ribose sugar in 100 mM phosphate buffer saline (PBS), pH 7.4 and incubated at 37º C for three weeks, followed by extensive dialysis against phosphate buffer saline to remove unbound constituents. LDL alone was served as control.

Various proteins like HSA, Hb, IgG and Poly amino acids like Poly-L-lysine, Poly-L-arginine and Poly-L-histidine were also modified by using D-ribose with same concentration and with the same procedure (Akhter et al., 2013).

4.2.4. Measurement of superoxide anion

To detect the presence of $O_2^{-}$ anion in the LDL glycation mixture, a cytochrome-c reduction assay was performed. The reaction mixture contained D-ribose (80 mM) and 100 µM cytochrome-c in 20 mM phosphate buffer (pH 7.4). The reduction rate was determined as the increase in absorbance at 550 nm for 10 min at room temperature. Absorbance was taken at one min intervals (Beauchamp and Fridovich, 1971).
4.2.5. Measurement of hydroxyl radical

Detection of hydroxyl radicals was carried out by measuring TBA reactive 2-deoxy-D-ribose oxidation products. Reaction mixtures containing 80 mM D-ribose, and LDL (62.5µg/ml) was incubated at 37°C for two weeks. The degradation of 2-deoxy-D-ribose was measured by adding 1 mL of 2.8% (w/v) trichloroacetic acid, 1 mL of 1% (w/v) TBA followed by heating at 100°C for 10 min. After cooling the absorbance was read at 532 nm (Halliwell et al., 1981).

4.2.6. Immunization schedule

LDL (25µg), its D-ribose modified counterpart and D-ribose (25µg) were emulsified with complete Freund’s adjuvant. The complex and 500 µl CFA were injected intramuscularly in the hind leg muscles of rabbits (three animals in each group). Subsequent injections were given in incomplete Freund’s adjuvant until the period of study. Weekly injections of antigen (LDL/D-ribose modified LDL/D-ribose) were given for seven weeks and thus each animal received a total of 175µg antigen during the course of immunization. One week after the last dose of immunogen, blood was collected and put it at 37 °C for 2-3 hours. The serum separated and decomplemented by heating at 56 °C for 30 min on the water bath. Preimmune blood was collected prior to immunization. The serum was stored in small aliquots at -80 °C with sodium azide (0.1%) as preservative (Moinuddin et al., 2012).

4.2.7. Estimation of conjugate diene (CD) and thiobarbituric acid reducing substance (TBARS) in plasma

The blood from each NZW female rabbit in a given group was collected in heparinized tubes, for 2-3 h, mixed gently by inversion 2-3 times and incubated at 40 °C. Plasma was separated from blood by centrifugation at 2,500 rpm for 30 min, aliquoted and stored at –20 °C.
For the extraction of Lipid contents from plasma, the method of Folch et al. (1957) was employed. One volume of plasma was mixed with 5.0 volume of chloroform: methanol (2:1), followed by centrifugation at 1,000 rpm for 5 min to separate the phases. Most of the upper layer was removed; and 3.0 ml of the lower chloroform layer was recovered. The chloroform layer was placed in a test tube and incubated at 45°C till dryness. For the determination of conjugated diene (CD) in plasma, corresponding lipid residues were dissolved in 1.5 ml of cyclohexane and the absorbance was recorded at 234 nm against a cyclohexane blank in a Beckman DU 640 spectrophotometer. The concentration of conjugated diene formation was calculated by using a molar extinction coefficient of $2.52 \times 10^4 \, \text{M}^{-1}\text{cm}^{-1}$.

Lipid peroxide contents in plasma were assayed by the method of Yagi (1987). Plasma (25 µl) was mixed with 4.0 ml of 0.083 N H$_2$SO$_4$ followed by the addition of 0.5 ml of 10 % PTA. The samples were mixed and incubated for 5 min at room temperature and then centrifuged at 3,000 rpm for 10 min. The supernatant was discarded and the sediment was mixed with 2.0 ml of 0.083 N H$_2$SO$_4$ and 0.3 ml of 10 % phosphotungstic acid. The mixture was centrifuged at 3,000 rpm for 10 min, the sediment was suspended in 4.0 ml of water and 1.0 ml of TBA reagent (a mixture containing equal volumes of 0.67 % aqueous TBA solution and glacial acetic acid) was added. The reaction mixture was heated for 60 min at 95°C, cooled and the tubes were centrifuged at 3,000 rpm for 10 min. The absorbance of the supernatant was determined at 532 nm against a reagent blank in an Eppendorf Bio Spectrometer. The concentration of MDA was calculated by using a standard malondialdehyde (Liu and Lesca, 1982).

4.2.8. Enzyme linked immunosorbent assay (ELISA)
ELISA was performed on polystyrene plates (Shahab et al., 2012b) with slight modification. Polystyrene polysorb immunoplates were coated with 100
µl of the D-ribose, native or glycated LDL (10µg/ml) in 0.05 mol/l carbonate–bicarbonate buffer, pH 9.6. The plates were incubated for 2 hours at 37 °C and overnight at 4°C. Each sample was coated in duplicate and half of the plate, devoid of antigen coating, served as control. Unbound antigen was washed three times with TBS-T (20 mmol/l Tris, 150 mmol/l NaCl, pH 7.4 containing 0.05% Tween-20), and unoccupied sites were blocked with 2% fat-free skimmed milk in TBS (10 mmol/l Tris, 150 mmol/l NaCl, pH 7.4) for 6 hours at 37°C. After incubation, the plates were washed again three times with TBS-T. Test serum was added to antigen-coated wells and reincubated for 2 hours at 37°C and overnight at 4°C. Bound antibodies were assayed with anti-rabbit IgG-alkaline phosphatase conjugate using p-nitrophenyl phosphate (pNPP) as substrate. Absorbance (A) of each well was monitored at 410 nm on an automatic microplate reader, and the mean of duplicate readings for each sample was recorded. Results have been expressed as a mean of $A_{\text{test}} - A_{\text{control}}$.

### 4.2.9. Competitive ELISA/ cross reactivity

The antigen-binding specificity of the antibodies was evaluated by competitive ELISA and cross reactivity (Shahab et al., 2013a). In direct binding ELISA D-ribose, N-LDL and G-LDL (0–20µg/ml) were mixed with constant amount of induced respective sera. In cross reactivity varying amounts of inhibitors like native and D-ribose modified proteins (LDL, HSA, Hb, IgG) and amino acids (Poly-L-lysine, Poly-L-arginine, Poly-L-histidine) (0–20µg/ml) were mixed with a constant amount of IgG. The mixture was incubated at room temperature for 2 hours and overnight at 4°C. The immune complex thus formed was added to the wells, in place of serum. The remaining steps were same as in direct binding ELISA. Percent inhibition was calculated using the following formula:

$$\text{Percent Inhibition} = 1 - \frac{A_{\text{inhibited}}}{A_{\text{uninhibited}}} \times 100.$$
In order to perform the cross reactivity, firstly the IgG antibodies were isolated using protein-A-Agarose column, based on affinity chromatography.

### 4.2.10. Purification of antibodies

Immunoglobulin G (IgG) was affinity purified from preimmune and immune sera on a protein A-Agarose column (Mustafa et al., 2012). Serum (0.5 ml) diluted with equal volume of PBS (pH 7.4), was applied to the column (0.9x15 cm) pre-equilibrated with above buffer. The wash through was recycled 2–3 times. Unbound IgG was removed by extensive washing with PBS (pH 7.4). The bound IgG was eluted with 0.58% acetic acid in 0.85% sodium chloride (Goding, 1976). Three millilitre fractions were collected in a measuring cylinder already containing 1 ml of 1 M Tris–HCl, pH 8.5, and absorbance was recorded. The IgG concentration was determined considering 1.4 OD280 = 1.0 mg mammalian IgG/ml. The isolated IgG was then dialyzed against PBS (pH 7.4) and stored at -80 °C.

### 4.2.11. Histopathological study of kidney sections light microscopic analysis

Slices of kidney from rabbits immunized with native and modified LDL was fixed in 10% formalin, dehydrated with increasing strength of ethanol, cleared in xylene, and wax impregnated. Paraffin blocks of the kidney pieces were fixed to block holder to cut 5-µm thick sections. Sections were de waxed with xylene and hydrated using descending grades of alcohol. Hematoxylin (nuclei stain) and eosin (cytoplasmic stain) were used for staining. Again, the sections were dehydrated with ascending grades of alcohol and cleared with xylene before finally mounting in a mixture of distyrene, a plasticizer, and xylene (DPX) for microscopic observation (Shahab et al., 2012b).

### 4.2.12. Immunofluorescence analysis

5µm thick sections were cut with the help of a cryostat at -30°C. Sections were fixed with acetone on glass slides, incubated with anti-rabbit IgG
fluorescein isothiocyanate conjugate (FITC) obtained from Sigma (St. Louis, MO) for 30 min. After three wash with phosphate buffer saline (PBS, pH 7.4), the sections were mounted with 50% glycerol and viewed under fluorescent microscope (Shahab et al., 2012b).

4.3. RESULTS AND DISCUSSIONS

4.3.1. Modification of LDL by D-ribose

We previously demonstrated that in vitro treatment of commercially available LDL with D-ribose results in biophysical and biochemical alterations in LDL to form LDL-AGEs (Chapter 3; Ahmad et al., 2013).

The LDL concentration used in these experiments for glycation is 62.5 µg/ml. The average level of LDL in normal individual is 1 mg/ml, However the level of glycated LDL in diabetic subjects is 30 µg/ml. Therefore, to see the significant change in LDL macromolecule we used double the amount of glycated LDL in diabetic subjects which is 62.5 µg/ml.

4.3.2. Estimation of superoxide anion

Superoxide generation in the D-ribose modified human LDL was quantitated by a cytochrome C reduction experiment. During incubation of D-ribose with LDL, the formation of superoxide anion was gradually increased in time dependent manner. The incubation of D-ribose with LDL produced 30.5 nmol O2\(^\cdot\) \text{ml}^{-1} \text{h}^{-1} \text{ compared to 2.34 nmol O2\(^\cdot\) \text{ml}^{-1} \text{h}^{-1} \text{ with D-ribose alone. Superoxide dismutase (SOD) inhibited the superoxide radical generation. Upon increasing concentration of SOD, the superoxide generation was gradually decreased.}

4.3.3. Estimation of hydroxyl radicals

Incubation of 2-deoxy-D-ribose with D-ribose produced 14.2 nmol TBARS ml\(^{-1}\) however; reaction of LDL with D-ribose in the presence of Fe\(^{3+}\) enhanced it to 22.1 nmol TBARS ml\(^{-1}\). Radical scavengers like mannitol, catalase and a metal ion chelator, desferrioxamine significantly inhibited the production of
TBARS. TBARS generation was inhibited up to 58.3% when mannitol was used as a scavenger. However, catalase and desferrioxamine inhibited TBARS up to 52.4 and 60.2% respectively. The result suggests that D-ribose-mediated hydroxyl radical generation may be caused by traces of transition metals. Furthermore, it is also being suggested that the redox reactions of iron may facilitate the generation of hydroxyl radical by reaction of LDL and D-Ribose.

4.3.4. Estimation of CD and TBARS in plasma

CD in plasma was significantly increased in D-ribose glycated LDL induced plasma of NZW female rabbit in comparison preimmune plasma while moderately increased in D-ribose, CFA and N-LDL induced plasma (Table 4.1). TBARS in plasma was also significantly increased in D-ribose glycated LDL induced plasma in comparison to preimmune plasma while a very less or no change in D-ribose, CFA and N-LDL induced plasma (Table 4.1).

4.3.5. Immunogenicity of LDL and its D-ribose-modified form

In the present study, antigenicity of D-ribose, CFA, native and D-ribose-modified LDL was probed in New Zealand white (NZW) female rabbits. The result from direct binding ELISA D-ribose showed not significant binding, native LDL was moderately immunogenic eliciting average titer of antibodies, while its modified form i.e. glycated LDL was found to be much more immunogenic than its native form (Figure 4.1). CFA not showed significant binding with its induced sera (data not shown). Native LDL showed the titer
Table 4.1 Effect of CFA, D-ribose, N-LDL and G-LDL on plasma TBARS and Conjugated Diene (CD) in control and treated NZW female rabbits

<table>
<thead>
<tr>
<th>S.No.</th>
<th></th>
<th>Conjugated diene (CD)</th>
<th>TBARS</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Preimmune sera</td>
<td>15.15 nM ±0.041 nM</td>
<td>0.48 µM±0.044 µM</td>
</tr>
<tr>
<td>2.</td>
<td>CFA immunized sera</td>
<td>22.5nM±0.17 nM</td>
<td>0.044 µM±0.006 µM</td>
</tr>
<tr>
<td>3.</td>
<td>D-ribose immunized sera</td>
<td>27.22 nM ±0.73 nM</td>
<td>0.09 µM ±0.03 µM</td>
</tr>
<tr>
<td>4.</td>
<td>N-LDL immunized sera</td>
<td>27.38 nM ±0.13 nM</td>
<td>0.112 µM ±0.042 µM</td>
</tr>
<tr>
<td>5.</td>
<td>G-LDL immunized sera</td>
<td>54.76 nM ±0.088 nM</td>
<td>3.85 µM ±0.057 µM</td>
</tr>
</tbody>
</table>

Each data represents average of three experiments. The values represent the mean ±SD.

value of >1:3200 while D-ribose glycated LDL antiserum showed high titer antibodies (>1:25,600) in direct binding ELISA. Binding of the preimmune serum was of low magnitude (Figure 4.1).
Furthermore, in the competitive inhibition ELISA we used D-ribose glycated LDL antiserum to explore its specificity against the immunogen i.e. D-ribose glycated LDL (Figure 4.2).

The IgG antibodies exhibited wide range of heterogeneity in recognizing varied inhibitors that included native and glycated analogues of LDL, HSA, Hb, IgG, poly L-lysine, poly L-arginine and poly L-histidine. A maximum of 82.3% inhibition of the D-ribose-LDL-IgG antibody was observed with the immunogen, when it was used as an inhibitor (Figure 4.2). The antibody was highly specific for the immunogen as only 2.5µg/mL D-ribose modified-LDL caused 50% inhibition in the antiserum activity (Table 4.2). The IgG antibody showed considerably low recognition for native LDL, which caused a maximum inhibition of only 33.4%. The inhibition of D-ribose-LDL-IgG antibody by various competitors is given in Table 4.2. IgG concentration was kept constant (40µg/ml) in all experiments. The cross reactivity observed with various amino acids (poly L-lysine, poly L-arginine, poly L-histidine) and protein (HSA, Hb, IgG) was due to the recognition of antigenic determinants. Reports show that D-ribose induces maximum modification at arginine and lysine residue in modified LDL case (Table 4.2), which explains that the higher cross reactivity of antiserum with D-ribose-arginine and D-ribose-lysine. This result is corroborated with the previously published reports (Cervantes-Laurean et al., 1993).

Furthermore, a small spectrum of information like body weight, food consumption and illness were observed in phenotypic studies. In-life parameters we have monitored daily clinical observations, weekly body
Figure 4.1. Level of induced antibodies against D-ribose-modified LDL. Direct binding ELISA of D-ribose antisera (◻), N-LDL antisera (▲), G-LDL antisera (♦) and preimmune sera (■). The microtiter wells were coated with D-ribose, N-LDL and G-LDL (10µg/ml) in direct binding ELISA of D-ribose antisera, native LDL antisera and G-LDL antisera respectively. Each data represents average of three experiments. The values represent the mean ±SD.

Figure 4.2. Inhibition of serum antibodies against N-LDL and G-LDL binding by D-ribose (♦), N-LDL (▲) and G-LDL (■) respectively. The microtiter plate was coated with D-ribose, N-LDL and G-LDL (10µg/ml) respectively. Each data represents average of three experiments. The values represent the mean ±SD.

Table 4.2. Cross reaction of anti-D-ribose-modified LDL IgG

<table>
<thead>
<tr>
<th>S.No</th>
<th>Inhibitors</th>
<th>Maximum % inhibition At 20 µg/ml</th>
<th>Concentration for 50% inhibition (µg/ml)</th>
<th>Percent relative affinity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>D-ribose-LDL</td>
<td>82.3±0.71</td>
<td>2.5±0.04</td>
<td>100±0.04</td>
</tr>
<tr>
<td>2</td>
<td>Native LDL</td>
<td>33.4±0.15</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>3</td>
<td>D-ribose-HSA</td>
<td>46.22±1.34</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>4</td>
<td>Native HAS</td>
<td>32.15±1.03</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>5</td>
<td>D-ribose-Hb</td>
<td>52.03±1.74</td>
<td>18.4±0.12</td>
<td>13.59±0.25</td>
</tr>
<tr>
<td>6</td>
<td>Native Hb</td>
<td>24.21±0.78</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>7</td>
<td>D-ribose-IgG</td>
<td>48.101±0.86</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>8</td>
<td>Native IgG</td>
<td>23.17±0.23</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>9</td>
<td>D-ribose-Poly-L-lysine</td>
<td>56.8±1.81</td>
<td>15.3±0.22</td>
<td>16.34±0.26</td>
</tr>
<tr>
<td>10</td>
<td>Native Poly-L-lysine</td>
<td>27.27±0.32</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>11</td>
<td>D-ribose-Poly-L-arginiine</td>
<td>58.312±1.32</td>
<td>13.7±0.11</td>
<td>18.25±0.28</td>
</tr>
<tr>
<td>12</td>
<td>Native Poly-L-arginiine</td>
<td>18.27±0.21</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>13</td>
<td>D-ribose-Poly-L-Histidine</td>
<td>39.78±0.42</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>14</td>
<td>Native Poly-L-Histidine</td>
<td>26.022±0.32</td>
<td>---</td>
<td>---</td>
</tr>
</tbody>
</table>

Micro titer plates were coated with D-ribose-modified LDL (10.0 µg/mL). Each data represents average of three experiments. The values represent the mean ±SD.

weights. Up to 3rd week of immunization there was no phenotypic changes
were observed moreover no significant alterations were found in the total
daily food consumption and weekly food consumption without any illness.
We also observed that animals immunized with glycated LDL were more
prone to reduction in body weight & food consumption as composed to native
LDL.

Ribose is a naturally occurring pentose monosaccharide present in all
living cells including the blood and is a key component of many important
bio-molecules such as riboflavin, RNA and ATP (Kellar et al., 1988). Our
study has shown that D-ribose causes structural perturbations in LDL
molecule resulting in the generation of neo-antigenic epitopes which are
recognized as non-self by the immune system, thus breaking the immune
tolerance existing normally to self-antigens. Previously it has been shown that
oxidized form of the LDL is highly immunogenic and shows potential
recognition of auto-antibodies raised against modified LDL (Lopes-Virella et
al., 2011). This is the first study in which we explored the immunogenicity of
native and glycated LDL by D-ribose. Our results also point towards the
possible involvement of this pentose sugar in the induction of antibody
response in different diseases. Thus, it is reasonable to conclude that at
present the exact pathophysiological role of glycation of apo B is still obscure
and needs further study.

Vigorous humoral response in animals immunized with D-ribose-
modified LDL suggests an alteration in the LDL structure, resulting in the
generation of neo-epitopes, which are recognized as nonself by the immune
system, leading to the robust production of antibodies.

The half-life of LDL in the circulation is in the order of 3–4 days. Some
research groups experimentally showed on rabbits that in vitro glycated-LDL
was cleared at a slower rate than control LDL and thus stayed longer in the

circulation (vascular mean residence time: 10 vs 8 h, p less than 0.001). The bodies mean residence time for glycated-LDL was 22 h vs 17 h for control LDL. In diabetic animals the catabolic parameters of both LDL preparations changed towards a faster clearance, the effect being greatest for glycated-LDL (total mean residence times of glycated-LDL pre-diabetic: 19 h, diabetic: 16 h; control LDL pre-diabetic and diabetic: 14 h). The difference in clearance between glycated and control LDL was thus strongly reduced. This suggests an increased activity of the non-receptor mediated pathway in diabetes mellitus, possibly co-responsible for an increased atherosclerotic risk (Kortlandt et al., 1992).

In histological examination of kidney sections of study of NZW female rabbits immunized with native LDL showed a normal kidney morphology having normal glomerular, tubular, and vascular structure (Figure 4.3a). Whereas D-ribose-LDL immunized rabbits revealed hyper cellular and congested glomeruli with thickened basement membrane indicative of immune complex deposition (Figure 4.3b & Figure 4.3c). In the fluorescence microscopic examination, low fluorescence signal was detected in FITC-labeled kidney sections of rabbits immunized with native LDL (Figure 4.4a). However, kidney sections of D-ribose-LDL-immunized rabbits showed GBM having a series of intense fluorescence points (Figure 4.4b & Figure 4.4c). The deposition of immune complexes in the kidney points toward the possible involvement of D-ribose in glomerulonephritis.

4.4. CONCLUSION

Our research group and others too have shown that D-ribose causes structural perturbations in the LDL molecule (Ahmad et al., 2013) resulting in the metabolic abnormalities associated with glycation of LDL molecule that
Figure 4.3 (a) Kidney section from female rabbit immunized with native LDL showing normal morphology of glomeruli (Magnification-60x); whereas, (b) (Magnification-60x) and (c) (Magnification-60x) Kidney section from female rabbit immunized with D-ribose-modified LDL showing larger glomerular capillary tuft with increased number of nuclei showing proliferation of endothelium and mesangial cells. Scale bar 100µm.
Figure 4.4 (a) Immunofluorescence of kidney sections of rabbit immunized with native LDL; whereas (b) and (c) Immunofluorescence of kidney sections of rabbit immunized with D-ribose-modified LDL. Immune complex deposition on GBM is shown. Scale bar 100µm.

generation of oxygen free radicals, resulting in oxidative damage to both the lipid and protein components of LDL and to any nearby macromolecules. We have also indicated its possible role in glomerulonephritis, an aspect that Akhter F et al. (2014), PLoS ONE, 9(11): e113144.
needs further probe. Our results would pave the way for further studies to evaluate the role of AGE-LDL in the progression and pathophysiology of diabetes-mediated atherosclerosis include diminished recognition of LDL by the classic LDL receptor; increased covalent binding of LDL in vessel walls; enhanced uptake of LDL by macrophages, thus stimulating foam cell formation; increased platelet aggregation; generation of neo-antigenic epitopes which are recognized as non-self by the immune system, thus breaking the immune tolerance existing normally to self-antigens. Formation of LDL-immune complexes; and generation of oxygen free radicals, resulting in oxidative damage to both the lipid and protein components of LDL and to any nearby macromolecules. We have also indicated its possible role in glomerulonephritis, an aspect that needs further probe. Our results would pave the way for further studies to evaluate the role of AGE-LDL in the progression and pathophysiology of diabetes-mediated atherosclerosis.

Recently, the presence of auto-antibodies in diabetes mellitus patients against the glycated DNA was investigated by our group (Ahmad et al., 2014; Shahab et al., 2014). There are also couple of recent reports for the presence of the auto-antibodies in the sera of the cancer patients which needs to be validated further (Ahmad et al., 2011a). Therefore the need of the hour is to stop this dreaded reaction using novel approach like nanoconjugation (Rahim et al., 2013). Our research group has recently shown some medicinal plants that have potent antioxidant property like *Ficus virens* and *Boerhaavia diffusa* (Iqbal et al., 2013; Akhter et al., 2013). Eventually, based on its antioxidant and antidiabetic characteristics, it is hypothesized that these plants might exhibit antiglycating properties as well. This is just a hypothesis to prove the same to get rid of the anomalies happening due to the glycation reaction as discussed above. The finding present in this chapter would also help the way
for the treatment of diabetes and its secondary complications like retinopathy, neuropathy and nephropathy.