DISCUSSION

5.1 The LH-specificity in Patients with Type-2 Diabetic Mellitus

It is well known that the red blood cells of patients with type-2 diabetes mellitus contain variable amounts of glucose. We were interested to see that the agglutinability of diabetic red blood cells with a given lectin would differ or not from their normal counterparts. To serve this purpose, we tested diabetic and normal red blood cells with lectins extracted from *Phaseolus vulgaris* (black and red variety), *Dolichos biflorus*, *Erythrina lithosperma*, *Glycine max* (white and yellow variety) and *Pisum sativum*. None of the lectins except *Erythrina lithosperma* could differentiate between diabetic and normal A, B and O cells in regards to their titre agglutinability. For this reason, in the present work we have focused our attention only on the *Erythrina lithosperma* lectin.

As mentioned earlier, Shrivastava *et al.* (1979) reported a new red blood cell membrane specificity called by them LH in the seed extract of *Erythrina lithosperma*. They successfully used the anti-LH lectin to discriminate human red blood cells as LH-positive (firm agglutination) and LH-negative (weak agglutination) types. Considering the LH dimorphism, we have here used the anti-LH lectin to differentiate the diabetic and normal red blood cells.

The purpose of the study was to find out the difference in the LH specificity in patients with type-2 diabetes mellitus and controls. This hypothesis was generated on the assumption that in diabetic patients, persistent hyperglycemia either due to insufficient production of insulin by pancreas or improper utilization of the glucose, erythrocytes remained in hyperglycemic environment throughout their life span which led to significant changes in erythrocyte aggregation and deformability. Shin *et al.* (2007) reported that the cytoskeleton proteins of the erythrocytes from diabetic patients were heavily glycosylated. Garnier *et al.* (1990) and Labrouche *et al.* (1996) described that spectrin was oxidatively damaged several lipids (free cholesterol, sphingomeyelin and phosphatidylcholine) also on the outer surface of the phospholipid bilayer were significantly decreased. Because of excessive in vivo glycosylation on the cell
membrane of erythrocyte, there would be less number of attachment sites or receptors available to the carbohydrate-rich reactive component of the anti-LH lectin than would normal erythrocytes. Therefore, in patients with type 2 diabetes mellitus, there should be more LH-negative individuals than in the normal population.

The LH-specificity was studied by Sehajpal and Shrivastava (1981), Reddy et al (1981), Kaur and Shrivastava (1983) and Koley and Shrivastava (1992) in various populations. We have in present study, studied the LH-specificity or LH dimorphism in 261 patients with type-2 diabetes mellitus and 251 control samples from Amritsar, Punjab. Both these samples were adequately represented by the major four religious groups of Amritsar i.e. the Sikh, Hindu, Muslim and others. We designed our study to include

a) Comparisons between pooled diabetic and pooled controls
b) Inter-group comparisons among the diabetic samples
c) Inter-group comparisons among the controls
d) Comparisons between diabetic and control samples among the same religion

The results of the present study indicated that the patients with type-2 diabetes mellitus were overwhelmingly LH-negative, showing statistically significant differences (p<0.001) between them, and these differences were particularly noticeable in patients with the blood group B, AB and O. In the distribution of the ABO types, the differences between these two groups was found not significant (p>0.05). Both the groups were in equilibrium for the phenotypic distribution of the LH and ABO types (Table 4.1 and 4.2). As the LH and ABO systems seem to be associated, we incorporated these two systems in our study to obtain more meaningful results. As it was found that no sex-difference were established in the distribution of LH and ABO systems, the male and female samples were pooled for all the analyses. In regard to the distribution of the LH types with blood group B, AB and O, statistically significant differences were obtained (p<0.001) between the patients with type-2 diabetes mellitus and controls. But no significant differences were found statistically in regard to the distribution of the LH types with blood group A in these two groups.
A significant preponderance of LH type in diabetic patients with blood groups B, AB and O allowed us to conclude that the LH system had more than a chance association with diabetes mellitus. The glycosylated erythrocytes membranes of diabetic patients offer less number of attachment sites to the carbohydrate-rich reactive component of the anti-LH lectin than normal erythrocytes. The AB cells react weakly with the anti-LH lectin following the order O>B> AB>A and this might explain why we did not find any significant differences in the distribution of the LH types in patients and controls with blood group A.

When the patients were compared among themselves in regard to the distribution of the LH types, no significant differences were found. In the distribution of the ABO types, differences between the groups were not statistically significant (Table 4.15). As regard to the distribution of the LH types among patients with the blood groups A, B and AB, the differences observed were statistically significant (p<0.05) (Table 4.23).

Likewise, when we compared the distribution of the LH and ABO types and the distribution of the LH types with blood groups A, B and AB among the four religious groups which together made up the control samples, statistically significant differences (p>0.05) were not found.

Kornfeld and Kornfeld (1971) as well as Goldstein and Hayee (1977) have pointed out that lectins have the ability to bind with specific membrane receptors. Lectinological studies conducted by Bunn et al. (1978) and Mcfarland et al. (1979) as well as by Dolhofer and Weiland (1980) established the glycosylation of hemoglobin and other proteins in man. Much earlier, Bailey et al. (1976) had also suggested the possibility of glycosylation in erythrocyte membrane proteins. In this context, the opinion of McMillian et al. (1978) was also noteworthy i.e. changes in erythrocytes membrane could result in alteration of its properties.

In this connection, it might be relevant to note that lectins and antibodies were found to mimic the action of insulin in fat cells (Cautrecases, 1973; Cautrecases and Tall, 1973 and Kahn et al., 1977). Human erythrocytes have been reported to have two populations of insulin receptors, one of which is inhibited by lectin Concanavalin-A (Victoria et al., 1980). The anti-LH lectin may also possibly, react in the same manner.
In the same religious groups, when we compared the diabetic patients and controls, highly significant differences (p<0.001) were found in the Sikh patients and controls and the Hindu patients and controls (Table 4.47 and 4.50) and significant differences (p<0.05) were found in the other patients and controls in regard to the distribution of the LH types. Among the Muslim group, no significant differences (p>0.05) were found. The LH type was predominant in diabetic Sikh, Hindu and other groups.

The results of the previous studies indicated that, as against normal individuals, the patients with diabetes mellitus were significantly more LH negative (p<0.001) and also that this differences were particularly noticed in patients with blood group A and B. The findings of the present study followed the finding of previous studies (Sehajpal et al., 1981; Koley et al., 1990 and 1994)

5.2 Properties of the Anti-LH lectin

5.2.1 Hemagglutination activity

The crude extracts of the anti-LH lectin Erythrina lithosperma agglutinated all types of human erythrocytes tested. These findings were agreed with those of Allen and Brillantine (1969), Silvia et al. (1998) who described that the vast majority of lectins agglutinated in a non-specific way to all types of human red cells. Haemagglutination activity of the anti-LH lectin Erythrina lithosperma showed that the lectin was not specific to any blood group determinants i.e. they could agglutinate all blood types. This finding suggested that the lectin was a non-specific agglutinin of the three different blood group antigen receptors of the RBCs membrane or also called as the lectin recognize RBCs membrane receptors common to the three RBCs types. However titre value of B and O blood group cells were higher than A blood cells. This difference in titre value might be due to differences in the non-reducing ends of blood group determinants. A blood group determinants have N-acetyl glucosamine side group and B group determinants have galactose (both these sugars are specific to the lectin but N-acetyl glucosamine inhibits more), linked to the basic O group determinant, where there is no side group (Robert and Murray, 1999).
As against this non-specificity of the anti-LH lectin, many lectins have been shown to be specific agglutinins, like the anti-A from *Dolichos biflorus*, *Phaseolus limensis* and *Crotalaria lanceolata*, the anti-H from *Lotus tetragonolobus* and anti-M from *Vicia graminea* (Lis and Sharon, 1972). These lectins are important for blood typing, especially for distinguishing between different sub-groups within the A, B, O system of blood grouping (Palatink *et al.*, 1980). Like the anti-LH lectin, many lectins have been reported to be non-specific (Entlicher *et al.*, 1970; Bhattacharyya *et al.*, 1981 and Magdolina *et al.*, 1992) reported a non-specific lectin from *Pisum sativum*, *Erythrina suberosa* and *Sechium edule* respectively.

### 5.2.2 Effect of pH on the heamagglutination activity of the anti-LH lectin

The pH stability of the anti-LH lectin showed that the lectin was significantly stable from pH 5.2-7.0. However, the optimal pH for lectin binding was between pH 6.25-7.0. The stability of the extracts at various pH ranges demonstrated that these remained constant between pH 1.95 and pH 9.0 completely loosing hemagglutinating capacity at pH below 1.95 and above 9.0. Robertson and Strenght (1983) and Cammue *et al.* (1985) reported that the lectins from seed of *Vigna unguiculata* and the tubercle of *Eranthis hyemalis* maintained their hemagglutinating activity in a pH range of 2.0 to 12.0 for *Vigna* extract and 3.0 to 11.0 for the *Eranthis* extract. Nanne and Aragon (1991) found that the lectin of *Erythrina costaricensis* was stable at a pH range of 2.0 to 10.0.

Change in pH did affect the hemagglutinating activity of the anti-LH lectin. The results indicated that the lectin became totally inactive to react with red blood cell surface antigens when the pH was lowered to 1.25 and again at pH 7.0. At pH 1.25, perhaps there was a change in the membrane potential of erythrocytes which might be responsible for negative reaction. At high pH 7.0, almost all ions (Na⁺ and Cl⁻) presented in normal saline (major ingredients of lectin preparing medium) were dissociated which might interfere with the agglutination reaction of the anti-LH lectin on erythrocyte membrane surface receptors. Dissociation of ions at high pH could affect greatly the hemagglutination activity (Paulova *et al.*, 1971) and our results tend to support the hypothesis.
5.2.3 Effects of ions on the hemagglutination activity of the anti-LH lectin

Metal analysis of anti-LH by atomic absorption spectroscopy showed that the lectin contained Ca$^{2+}$ and Mg$^{2+}$ cations. Demetalization of the anti-LH lectin removed these cations in appreciable amount, but complete removal was not achieved. It was noted that demetalization of the anti-LH lectin did not affect its agglutinating capacity, but it resulted in decreased thermal stability. This suggested that metal ions were not essential for agglutination. Ahmed and Chatterjee (1989) reported that treatment of jacalin by acetic acid and EDTA, neither affected the hemagglutinating activity of the lectin nor did incorporation of Ca$^{2+}$ and/or other bivalent ions in saline showed any enhancement in the haemagglutination titre. Doyle et al. (1976) reported that metals confer a high degree of structural stability to Con-A lectin, protecting the lectin against heat inactivation and hydrolysis by proteolytic enzymes.

5.2.4 Effects of temperature on the stability of the anti-LH lectin

The anti-LH lectin was incubated at different temperature ranged between 30°C-100°C. The anti-LH lectin treated with temperature like 30-50°C, showed best hemagglutination activity. At 60°C, the anti-LH lectin did react weakly and at 80°C, it completely lost the hemagglutination activity. Like other lectins, the anti-LH lectin was also thermostable to some extent. It was stable up to 60°C. Thermal stability data showed that the stability of the anti-LH lectin was temperature dependent. Ahmed and Chatterjee (1989) reported that jacalin was stable at 50°C, the heat stability was temperature dependent. Indravathamma and Seshadri (1984) and Bose and Bhalia (1989) studied that temperature exhibited a limited range of variability in the agglutination pattern of lectins against human erythrocytes.

Now it is well established that lectins interact with cell surfaces through their saccharide determinants. Watkins and Morgan (1952) gave the opinion that the structure of sugars closely resembled with the specific red cell receptors and that the active sites on the agglutinins were blocked by the specific sugars, causing inhibition of hemagglutination. The inhibition of lectins by different kinds of polysaccharides was studied by Morgan and Watkins (1953), Krupe (1953 and 1956), Makela (1957), Bhatia and Allen (1962), Bhatia and Boyd (1962), Goldstein and So (1965), Lloyd et al.
(1969), Pardoe and Unlenbruck (1970), Sharon and Lis (1972) and many other scientists.

5.2.5 Sugar inhibition assay of the anti-LH lectin

Haemagglutination inhibition studies showed that, of the sugars tested, N-acetyl glucosamine was the most potent inhibitory sugar followed by galactose, lactose, raffinose, mallibiose and sucrose. Other sugars tested were found to be incapable of inhibiting haemagglutination. Hammarstorm et al. (1977) reported that a number of lectins displayed preferential affinity to N-acetyl glucosamine but also reacted with galactose. Conversely, there was a group of lectins that displayed a primary specificity for galactose and cross reacted with varying degree to N-acetyl glucosamine. For example, Hayes and Goldstein (1974) reported that lectin from B. simplificofilia and Nicolson et al. (1974) reported lectins from Ricinus communis were specific to N-acetyl glucosamine, did also bind to galactose, where as some others lectins like PNA interacted with galactose only (Pereira and Kabat, 1974).

Makela in 1957, reported that 4-hydroxy group was critically involved in lectin binding, so that lectin which did bind glucose did not interact with galactose and vice-versa. This observation supported the finding that, binding of anti-LH lectin was not inhibited by glucose but were inhibited by galactose, which differed from glucose only at the C-4 atom.

N-acetyl glucosamine, galactose, lactose, raffinose, mallibiose and sucrose inhibited the agglutinating activity of crude extracts of the anti-LH lectin in human red blood cells. Of these carbohydrates, N-acetyl glucosamine and galactose were the most potent inhibitors. The inhibitory activity of raffinose of hemagglutination by the anti-LH lectin extract could explain by the recognition of the galactose moiety of this trisaccharide. Lis et al. (1985) proposed that the differences in behavior of the different lectin extracts with rabbit red cells and mononuclear human cells was caused by variations in or close the combination site of the carbohydrate to the red cell membrane. All of the Erythrina extracts that have been studied in the past by various authors, showed the same pattern of inhibition with these carbohydrates (Bhattacharyya et al., 1981; Lis and Sharon, 1985 and Nanne and Argon, 1991).
5.2.6 **Estimation of molecular weight of the anti-LH lectin**

The homogeneity of lectin was checked by PAGE. On SDS-PAGE, after reduction with β-mercaptoethanol, the anti-LH lectin migrated as a two band having a molecular weight of 26 kDa and 28 kDa. This showed that the native anti-LH lectin was composed of two subunits, which were linked by disulphide bonds. The linking of subunits was not by non-covalent bond and or electrostatic attraction, since the monomers were not dissociated in the presence of SDS. If the monomers were linked by non-covalent and or electrostatic attraction, the monomers would dissociate in the presence of SDS, but in the anti-LH lectin, dissociation of subunits was not observed in SDS-PAGE. On the other hand, reduction of the anti-LH lectin with β-mercaptoethanol, reduced disulphide bonds, which linked the monomers. So, when the lectin was reduced with β-mercaptoethanol, the subunits got dissociated, having a molecular weight of 26KDa and 28KDa, which was half the molecular weight of the native lectin. Thus the anti-LH lectin was composed of two identical subunits of molecular weight of 26 and 28 KDa, linked together by disulphide bonds.

Bhattacharyya *et al.* (1981) purified non-specific D-galactose binding lectins from some *Erythrina* species. Iglesias *et al.* (1982) reported a D-galactose/N-acetyl-D-galactosamine specific lectin was isolated from *Erythrina crista galli* and it agglutinated human erythrocytes of the blood types as well as rabbit erythrocytes.

Many researchers have used gel chromatography and SDS-PAGE for the determination of molecular weight of lectins. Magdolna *et al.* (1992) used SDS-PAGE and Sephadex G-100 gel chromatography for the determination of molecular weight of *Sechium ehle* lectin. They reported that this lectin had a molecular weight of 44 KDa by PAGE, but on SDS-PAGE under reduced condition, the lectin showed a single band of 22 KDa. Thus they suggested that the subunits were non-covalently linked. Lotan *et al.* (1975c) observed similar results in *Arachis hypogaea* lectin.

5.3 **Association of HbA1c with Lipid Profiles in Patients with Type-2 Diabetes Mellitus**

Lipid abnormalities are common in diabetic patients and frequently seen in patients with type-2 diabetic mellitus. The abnormal lipid profile observed in type-2
diabetes mellitus was said to be related to insulin resistance as reported in previous studies, which led to increased release of free fatty acids from fatty tissue, impaired insulin dependent muscle uptake of free fatty acids and increased fatty acid release to the hepatic tissue (Boden, 1997) which has been closely associated with diabetic dyslipidemia, hypertension (Mgonda et al., 1998) and enormous risk to cardiovascular diseases. Chronic hyperglycemia causes glycation of apolipoproteins and interferes with the normal pathways of lipoprotein metabolism (Carmena, 2008). Diabetic patients have much complications which includes elevated levels of LDL-C and triacylglycerol and low level of HDL-C. In the present study, the results showed that the lipid profiles were higher in diabetic patients and that they were in agreement with the findings of Wexler et al. (2005). The study revealed that dyslipidemia was observed in the diabetic population, but that HDL-C and LDL-C were not significantly decreased or increased respectively. Lipoprotein lipase, an insulin dependent enzyme which together with insulin resistance leads to increase in TG levels, results in type-2 diabetes mellitus having high levels of TG; HDL-C levels may be further reduced in diabetes mellitus due to elevated hepatic lipase activity that catalyzes HDL-C (Harno et al., 2008). In the present study there was highly positive significant corrections were observed between HbA1c and lipid profiles (TC, TG and LDL-C). Erciyas et al. (2004) also reported the positive correlation between HbA1c and TC, TG in patients with type-2 diabetes mellitus. The diabetic complications and control trial (DCCT) established HbA1c as the gold standard of glycemic control. The HbA1c value <7.0% reduced the risk of cardiovascular diseases and value >7.0% leads to dyslipedemia to the patients. (Rohlfling et al., 2002). Controlling the glycemic levels may significantly decrease the risk of cardiovascular diseases in diabetes mellitus. Khaw et al. (2001) has reported that reducing the HbA1c level by 0.2% could lower the mortality by 10%, thus the present study suggested the importance of glycemic control in prevention of cardiovascular diseases in type-2 diabetes mellitus.

It was reported in the present study that the patients with type-2 diabetes mellitus having HbA1c >7.0% were predominantly LH-negative type. It further supported our hypothesis.
5.4 **Strength of the Study**

1. To the best of our knowledge, the present study was the first of its knowledge reporting the LH-specificity in the patients with type-2 diabetes mellitus considering large sample size from north India which was the unique criteria of the study.

2. A wide range of very recent references incorporated in the present study was the real strength of the study.

3. The findings of the present study would be helpful to the patients with type-2 diabetes mellitus, medical personnel dealing with diabetic persons and the researchers involved in this particular field about the identification and characterization of the lectin *Erythrina lithosperma*.

4. The findings of the present study can be used in the field of sports nutrition, as the molecular level information regarding the nutrients would be useful to the athletes to select their foodstuff.

5. A vast number of properties of the anti-LH lectin studied in the present study were the novel part of the study.

5.5 **Limitation of the Study**

1. The samples were collected from Amritsar only.

2. More number of lectins would be incorporated in our future studies.

3. Apart from seeds, other parts of the plants would be used to prepare different lectins.

4. Besides the patients with type 2 diabetes mellitus, other diseases could have been tried to search any specificity with the lectin *Erythrina lithosperma*. 