CHAPTER V

SUMMARY
The present study was undertaken to systematically investigate the biochemical alterations in reticulocytes and erythrocytes, and alterations during the maturation of reticulocytes to erythrocytes in type 2 diabetic patients.

Based on the preliminary survey of male NIDDM patients of around 40-55 years with disease history of around 5-15 years, 20 treated but uncontrolled type 2 diabetic volunteers who had levels of blood glucose (188.47±23.50 mg%) and GHb of erythrocytes (3.25 ±0.67 μ moles of fructose equivalents/g Hb) well above the normal range were selected for the study. Age matched healthy males were served as controls.

Variations observed in hematological parameters, i.e., Hb content, RBC count, Packed Cell Volume, Mean Cell Hemoglobin, Mean Cell-Hemoglobin Content and Mean Cell Volume, and osmotic fragility of erythrocytes of type 2 diabetics indicated the presence of hypochromic macrocytic hemolytic anemia. The deficiency of serum iron as a cause for the development of anemia has been ruled out since, these patients showed no significant change in serum iron levels.

Reticulocytes and erythrocytes were separated from the whole blood samples and used for further studies.

Reticulocytes and erythrocytes were found to contain higher levels of GHb compared to corresponding cells of controls and diabetic
erythrocytes have 1.6 fold more increase in GHb level compared to diabetic reticulocytes. Erythrocytes contain higher levels of GHb compared to reticulocytes in both controls and diabetics with 1.7 fold more maturation dependent increase in diabetic patients.

Lipid peroxidation was assessed by measuring the TBA reactive products and it is found to be increased in plasma, reticulocytes and erythrocytes of diabetics compared to controls suggesting the existence of enhanced oxidative damage under diabetic conditions. The diabetic erythrocytes showed 4.5 fold greater extent of lipid peroxidation compared to diabetic reticulocytes indicating higher susceptibility of diabetic erythrocytes to oxidative damage than the diabetic reticulocytes. Maturation dependent decrease in lipid peroxidation was observed both in controls and diabetics. However, the decrease was found to be less in diabetics compared to controls. This is due to the differential extent of diabetes induced lipid peroxidation in reticulocytes and erythrocytes of NIDDM patients.

Diabetic patients showed increase in cholesterol and phospholipid content in plasma and membranes of reticulocytes and erythrocytes compared to controls. Diabetic erythrocytes contained more membrane cholesterol and less phospholipids compared to diabetic reticulocytes. This caused increased Chol/PL ratio of diabetic erythrocytes and decreased Chol/PL ratio in diabetic reticulocytes compared to that of controls. As reticulocytes mature to erythrocytes both membrane
cholesterol and phospholipids were found to be diminished in both controls and diabetics with more percent decrease in diabetics. The maturation dependent increase in Chol/PL ratio was found to be 3.1 fold more in diabetics compared to controls.

The activities of membrane bound and SH dependent enzymes i.e., acetylcholinesterase, Na\textsuperscript{+}-K\textsuperscript{+} and Ca\textsuperscript{2+},Mg\textsuperscript{2+}-ATPases were found to be decreased in both reticulocytes and erythrocytes of diabetic patients. The decrease was more pronounced in diabetic erythrocytes compared to diabetic reticulocytes. Corresponding to the decrease in ATPase activities, increased Na\textsuperscript{+} and Ca\textsuperscript{2+} and decreased K\textsuperscript{+} concentrations were observed in diabetic reticulocytes and erythrocytes with concomitant decrease in Na\textsuperscript{+} and Ca\textsuperscript{2+} and increase in K\textsuperscript{+} concentrations in plasma of diabetics. The alterations in cellular ionic concentrations were more pronounced in diabetic erythrocytes compared to diabetic reticulocytes. Maturation of reticulocytes to erythrocytes resulted in decrease in the activities of these membrane bound enzymes in both controls and diabetics with greater percent decrease in these enzyme activities in diabetics.

Levels of membrane protein bound hexose were found to be increased and membrane protein bound hexosamine and sialic acid were found to be decreased in diabetic reticulocytes and erythrocytes compared to controls. These changes in membrane protein bound carbohydrates were further more pronounced in diabetic erythrocytes compared to diabetic reticulocytes. As reticulocytes mature to
erythrocytes membrane protein bound hexosamine and sialic acid were found to be decreased and hexose was found to be increased in both controls and diabetics. These alterations in membrane protein bound carbohydrates were further more pronounced in diabetics.

Among the endogenous antioxidants present in the biological system, the GSH occupies a very important position. Decreased levels of cellular GSH were found in reticulocytes and erythrocytes of diabetics compared to that of controls, with more percent decrease in diabetic erythrocytes compared to diabetic reticulocytes. Maturation of reticulocytes to erythrocytes resulted in reduction in cellular GSH levels both in controls and diabetics. But this decrease was more pronounced during maturation of reticulocytes under diabetic conditions.

The activities of GSH utilizing (antioxidant) enzymes i.e., GSHPx and GST and GSH regenerating enzymes i.e., GR and G6PDH and glycolytic enzymes i.e., hexokinase and LDH were found to be decreased both in reticulocytes and erythrocytes of type 2 diabetic patients. Further, reduction in the activities of these enzymes was more pronounced in diabetic erythrocytes compared to diabetic reticulocytes. Maturation of reticulocytes to erythrocytes resulted in decrease in the activities of these enzymes both in controls and diabetics. This maturational dependent decrease was more pronounced in diabetic patients compared to controls.
Unlike the GSH dependent antioxidant enzymes the activity of catalase was found to be increased in both reticulocytes and erythrocytes of diabetics compared to controls. This increase was more pronounced in diabetic reticulocytes compared to diabetic erythrocytes. Maturation of reticulocytes to erythrocytes caused decrease in the activity of catalase in both controls and diabetics and this decrease was more pronounced in diabetics compared to controls.

In order to understand whether the alterations observed in type 2 diabetic patients were mainly due to hyperglycemia or due to other complex processes that prevail in type 2 diabetic patients, *in vitro* hyperglycemic studies were performed.

Suspensions of reticulocytes and erythrocytes (15%v/v) in phosphate buffered saline were incubated in the media containing varying concentrations of glucose i.e, 5-45mM (5, 15, 25, 35 and 45mM) in shaking water bath at 37°C for 15h. The reticulocyte/erythrocyte suspension containing 5 mM glucose is considered as control because human normal blood glucose level falls in the range of 4-6 mM. The cells harvested after the incubation period were used for the study of membrane lipid peroxidation, levels of cellular GSH and activities of enzymes involved in regeneration (GR and G6PDH) and utilization (GSHPx and GST) of GSH, catalase and glycolysis.
Effect of *in vitro* hyperglycemia on lactate production was studied with the same procedure in erythrocytes with an incubation period for 1 h.

The extent of lipid peroxidation was found to be increased as concentration of glucose in the incubation medium increased both in erythrocytes and reticulocytes with greater percent increase in lipid peroxidation at each concentration of glucose in erythrocytes compared to reticulocytes. These results are well correlated with those of NIDDM patients.

Unlike the results obtained with NIDDM patients, cellular GSH, the GSH dependent defense enzymes i.e., GSHPx and GST and GSH regenerating enzymes i.e, GR and G6PDH were found to be increased in both reticulocytes and erythrocytes with increased glucose concentration up to 25 mM and then decreased at 35 and 45 mM glucose concentrations. Similar trend was found regarding the glycolytic enzyme activities (hexokinase and LDH) of erythrocytes incubated at enhanced glucose concentrations. Similar to *in vivo* studies the percent decrease in the activities of all enzymes were greater in erythrocytes than reticulocytes. But the activity of catalase was found to be increased at all elevated levels of glucose in both reticulocytes and erythrocytes with less percent increment in the activity at 35 and 45 mM glucose concentrations.
Conclusions:

1. It is concluded from the present study that type 2 diabetic patients have hypochromic macrocytic hemolytic anemia.

2. The observed increased levels of GHb in both reticulocytes and erythrocytes indicated enhanced NEG of proteins and defective unloading of oxygen by the erythrocytes to the tissues and more pronounced glycation of hemoglobin of erythrocytes compared to reticulocytes may be due to longer exposure period of erythrocytes (~120 days) to hyperglycemia compared to that (~1-2 days) of reticulocytes.

3. The enhanced lipid peroxidation of erythrocytes and reticulocytes of type 2 diabetics indicates the presence of oxidative stress in these patients. The observed greater extent of lipid peroxidation in plasma compared to reticulocytes and erythrocytes indicates the oxidative damage of other tissues in NIDDM patients.

4. The observed enhanced lipid peroxidation in reticulocytes and erythrocytes of NIDDM patients might have resulted in significant alterations in composition and function of their membranes, activities of membrane bound enzymes, defects in antioxidant defense system and decrease in enzyme activities of glycolytic pathway. All these adverse effects identified in type 2 diabetic patients were found to be greater in erythrocytes compared to reticulocytes. Erythrocytes being metabolically less active and aged cells compared to reticulocytes are subjected to more alterations in both in vivo and in vitro hyperglycemia.
5. The decreased levels of GSH observed in reticulocytes and erythrocytes of diabetic patients indicate defective antioxidant system in these patients.

6. Maturation of reticulocytes to erythrocytes resulted in decreased GSH content and decreased activities of membrane bound enzymes (ATPases and acetylcholinesterase), antioxidant enzyme activities and enzymes involved in carbohydrate metabolism indicate that erythrocytes have less scavenging capacity and energy production than reticulocytes. This process is further intensified in type 2 diabetic patients.

7. From the data obtained from in vitro studies it is evident that hyperglycemia indeed has its effect over alterations observed in NIDDM patients. However, the healthy reticulocytes and erythrocytes used in in vitro studies require more concentrations of glucose in vitro i.e., 35 and 45 mM to show the adverse effects which were observed in NIDDM patients having glucose concentration of ~10.4 mM in the present study. Because of the inherent protection mechanisms present in healthy cells (reticulocytes and erythrocytes) which are produced under normal conditions (at 4-6 mM), when exposed to high glucose (15 and 25 mM) concentrations than that prevailing in the NIDDM patients (10.4 mM) in the present study have tried to encounter the existing oxidative stress induced by in vitro hyperglycemia.

Nephropathy, one of the long term complications found in diabetics may cause defective synthesis of the hormone erythropoietin a
regulator of erythropoiesis. This may lead to production of defective reticulocytes and erythrocytes in NIDDM patients.

8. Due to the production of defective reticulocytes and erythrocytes and exposure of these cells to hyperglycemic conditions in NIDDM patients additively caused failure of defense systems and malfunctioning of membrane bound enzymes. This may further lead to increased osmotic fragility of erythrocytes leading to hemolysis of these cells even at 10.4 mM glucose concentrations prevailing in NIDDM patients in the present study.

9. Since RBCs are found to behave as expendable scavengers competing with other tissues for the toxic effects of free radicals and protecting the host in vivo by neutralizing the exo and endogenous free radicals (Richards et al 1998). It may be suggested from this that erythrocytes under diabetic conditions with decreased scavenging efficiency might have shown its effect on overall protection of other tissues from oxidative damage in these patients.

It is concluded from the present study that significant alterations were observed in composition and enzyme activities during the maturation of reticulocytes to erythrocytes in normal human subjects. These alterations are further intensified in a direction leading to early senescence of erythrocytes in type 2 diabetic patients.

Due to the decreased production of ATP and NADH/NAD+ ratio from glycolytic pathway, NADPH from HMP shunt and enhanced non-
enzymatic glycation of proteins in these diabetic reticulocytes and erythrocytes rendering them more vulnerable to oxidative stress. This sequence of events ultimately affects the integrity of red cells by reducing the lifespan in diabetic condition associated with early senescence and anemic state in these patients. Further from the in vitro studies it is evident that hyperglycemia is also one of the factors for adverse effects identified in reticulocytes and erythrocytes of type 2 diabetic patients.