CHAPTER : 4

Effect of alloxan-diabetes on erythrocyte membrane structure and function in male and female rats.

Introduction

Diabetes mellitus is a disease characterized by chronic hyperglycemia and glycosurea produced by an absolute or relative deficiency of insulin (1). Exposure to increased glucose concentration leads to non-enzymatic glycosylation of proteins (2). Measurement of Hb_{A1C} as well as of other circulating glycated proteins, has thus become incorporated in to the clinical armamentarium for monitoring glycemic control in diabetic patients (2). This is because the extent of glycation is primarily dependent on the ambient glucose concentration that has prevailed within the erythrocyte or in the plasma during an interval of several weeks before the sample is taken (2).

A variety of polypeptide hormones including insulin can provoke rapid changes in phospholipid metabolism in the target tissue (3-9). Shifts in membrane phospholipid content may be important in regulating the activity of a variety of membrane enzymes (10). Changes in the dynamic properties of the cell membrane i.e. membrane fluidity, could be one of the events regulating in the altered insulin action (11) because a certain membrane fluidity seems to be necessary for the membrane proteins to function appropriately (12). It has been hypothesized that changes of membrane lipid structure and microviscosity may play the role of hormonal information transduce (13). There are several reports on fatty acid composition of blood cell membranes in human diabetics and

their unsaturated to saturated ratios but few studies have been devoted to phospholipid composition (14,15). Increased blood viscosity (16), altered red blood cells (RBC) aggregation (17) and increased adhesion of erythrocyte to endothelial cells (18) in diabetes have been reported. Active participation of blood cells is thought to be involved in vascular complications and diabetics present disorder in the function of blood cells (19).

Lipids are essential structural components in the membrane structure of cell (20). The cholesterol to phospholipid (C/P) ratio in the erythrocyte membrane is usually regulated to maintain proper membrane fluidity for normal functioning of the cell (20). Since erythrocytes have relatively insignificant degree of cholesterol synthesis (20), the exchange of cholesterol between plasma and erythrocytes is very active both in vivo and in vitro (20).

Many references are available on human blood cell membrane lipid, phospholipid and cholesterol content and membrane fluidity (21-25), while negligible work is done in other membranes. The opposite is true for animal models i.e. rat (21-25).

Studies were therefore carried out to measure the extent of glycosylation of erythrocyte membrane protein and serum proteins in animal model i.e. rat. Total phospholipid (TPL), cholesterol (CHL) content, content of phospholipid classes

and membrane fluidity of the erythrocyte membrane were studied. Rats of both the sexes were taken for this study. The parameters mentioned above were checked in both alloxan diabetic and insulin treated alloxan diabetic rats in comparison with control animals.

Materials and methods

Animals

Male and female albino rats of Charles-Foster strain weighing between 180 - 220 were used for these studies. After fasting for 24 h. the animals were given subcuataneously (s.c.) injection of freshly prepared alloxan using saline (0.9 % w/v NaCl) as a vehicle at a dose of 12 mg / 100 g body weight. (26). The control animals received only saline vehicle. The diabetic state was ascertained in terms of loss of body weight, polyurea, glycosurea, polydipsia, polyphagia and blood glucose levels (27). The animals were killed after 30 days of alloxan/saline treatment. One group of animals was used as a diabetic group. A group of diabetic animals received NPH insulin (Knoll Pharmaceuticals Ltd. India) s.c. at a dose of 0.8 U per 100 g body weight twice daily for seven consecutive days starting at the end of 22 days of alloxan treatment (27); the last injection was given 12 h prior to killing. This group was used as insulin-treated diabetic group.

Experimental procedures

At the end of one month after treatment with alloxan or vehicle, blood was collected in the test tubes containing trisodium citrate (final concentration 10 mM) and the tubes were centrifuged at 475 X g for 8 min. Supernatant and the buffy coat overlying the RBC pellet was discarded and the pellets were washed twice with 0.9 % NaCl. The washed RBCs were used for preparation of membrane (28, 29).

For isolation of serum, blood was collected in test tubes and was allowed to clot at room temperature for 1 hr. The tubes were then centrifuged at 475 X g for 8 min in a clinical centrifuge. The straw colored supernatant was carefully decanted.

Preparation of RBC membranes(28,29), determination of protein glycosylation (30), estimation of protein (31) were performed as described in the Chapter 2 of the thesis. Extraction of membrane lipids (32, 33), separation of membrane phospholipid classes (34), and measurements of membrane fluidity (33, 35), phospholipid phosphorous (36), cholesterol content (37) were performed as described in Chapter 3 of the thesis.

Results

Data in Table 1 show that in the male diabetic rats the body weight decreased (about 26 %), which was restored after

Table 1. Diabetes parameters in male rats.

Control(16)	Diabetic(16)	Insulin treated diabetic (13)
Body weight (g)	247.8±9.4	182.6±5.9 [a]	245.2±6.4 [#]
Blood sugar mM	5.9±1.6	24.4±1.9 [a]	4.8±0.3 [#]
Urine volume/ day (ml)	2.5±0.2	43.3±5.4 [a]	2.2±0.4 [#]
Sugar mg/ml urine	N.D.	26.3±1.6	N.D.
Sugar excretion g/day	--	1.10±0.09	--

Methods for blood glucose estimation and urine glucose estimation were as described in chapter 3 of the thesis. Results are expressed as mean±S.E.M. of the number of independent observations indicated in the parentheses.

N.D.= Not detectable

a, $P<0.001$ compared with corresponding control.

#, $P<0.001$ compared with corresponding diabetic.

insulin treatment. In diabetic male rats polyurea, glycosurea and hyperglycemia were observed. Compared to the controls the urine volume increased by 17 fold and the diabetic animals excreted more than 1 g sugar per day. In control and insulin treated diabetic groups urine sugar was not detectable. In the control group the blood glucose was 5.9 mM which increased by 4 fold in the diabetic group. In insulin treated diabetic rats the glucose value (4.8 mM) became comparable to the controls.

Practically similar trend was observed for the female rats (Table 2). However certain differences from the male group were noted. Thus insulin treatment could only partially restore the body weights. The basal blood sugar level in control group was somewhat higher compared to corresponding male group. Diabetic state caused 5.5 fold increase in the blood sugar level and in spite of insulin treatment the level remained elevated by about 20 % compared to the control group. The urinary sugar levels and daily urinary excretion of sugar were somewhat low compared with the male diabetic group (eg. see Table 1).

Physiologic differences between the male and female rats became evident even with respect to the extent of glycosylation of the erythrocyte membrane and serum proteins. Thus in the male rats (Table 3) the diabetic state did not influence the extent of erythrocyte membrane glycosylation but insulin treatment caused a significant decrease. On the

Table 2. Diabetes parameters in female rats.

Control(16)	Diabetic(16)	Insulin treated diabetic (12)
Body weight (g)	243.1±7.2	173.5±6.4 [b]	220.8±10.2 [#]
Blood sugar mM	6.7±0.49	37.2±4.4 [b]	8.1±0.33 [a,#]
Urine volume/ day (ml)	2.9±0.4	47.0±4.1 [b]	3.1±0.45 [#]
Sugar mg/ml urine	N.D.	15.3±0.63	N.D.
Sugar excretion g/day	--	0.76±0.07	--

Methods for blood glucose estimation and urine glucose estimation were as described in Chapter 3 of the thesis. Results are expressed as mean±S.E.M. of the number of independent observations indicated in the parentheses.

N.D. = Not detectable

a, $P<0.05$; b, $P<0.001$ compared with corresponding control.

z, $P<0.001$ compared with corresponding diabetic.

Table 3. Glycosylation of erythrocyte membrane and serum proteins.

		Control (8)	Diabetic (8)	Insulin treated diabetic (7)
Erythrocyte membrane (µg/mg protein)	Male	1212.10±189.89	1268.92±187.63	452.39±70.17 b,#
	Female	265.14± 20.24	814.42±140.10 c	251.60±64.41 @
Serum (µg / ml serum)	Male	3.92± 0.39	15.68± 0.59 d	8.23± 0.88 d,$
	Female	11.14± 0.32	12.44± 0.37 a	8.60± 0.87 a,#

Results are expressed as mean±S.E.M. of the number of independent observations indicated in the parenthesis.

a, $p<0.02$; b, $p<0.005$; c, $p< 0.002$ and d, $p<0.001$ compared with corresponding control.

@, $p<0.005$; #, $p<0.002$ and $, $p<0.001$ compared with corresponding diabetic.

other hand, the extent of glycosylation of serum proteins increased significantly (4 fold) in diabetes and insulin could only partly restore the same (Table 3).For the female rats the picture was different in that glycosylation of erythrocyte membrane proteins but not of serum proteins increased in diabetic state, insulin treatment partly restored the situation (Table 3).

The erythrocyte membrane lipid composition in the males and females from the control group was more or less comparable (Tables 4 and 5). Diabetic state caused an increase in the TPL content with the extent of increase being higher for the males. The CHL content decreased in males but increased in females. Insulin treatment could only partly decrease the TPL content in both the groups but effect on CHL content were opposite; in the males the CHL content continued to increase while in the females there was a slight decrease (Table 4 and 5). The TPL/CHL molar ratio increased by 4 fold in the diabetic male and this value could be restored by insulin treatment (Table 4). By contrast, the changes for the female rats were not so dramatic (Table 5).

Data in Table 6 show phospholipid composition of erythrocyte membranes from male rats. Thus in the control male rats phosphatidylcholine(PC) and phosphatidylehenolamine (PE) were the major components (44.18 and 25.31 % respectively), while lysophosphotidicacid (Lyso), sphingomyelin (SPM),

Table 4. Total phospholipid and cholesterol content of erythrocyte membranes from male rats.

	Control (11)	Diabetic (7)	Insulin treated diabetic (7)
Total phospholipid (TPL) (µg / mg protein)	494.04±25.03	1440.87±90.74 b	981.34±38.54 b,#
Cholesterol (CHL) (µg / mg protein)	200.21±19.16	147.23±12.10 a	458.58±28.50 b,#
TPL / CHL ratio	1.20± 0.05	4.79± 0.08 b	1.07± 0.04 #

Results are expressed as mean±S.E.M. of the number of independent observations indicated in the parentheses.

a, $p<0.05$ and b, $p<0.001$ compared with corresponding control.

#, $p<0.001$ compared with corresponding diabetic.

Table 5. Total phospholipid and cholesterol content of erythrocyte membranes from female rats.

	Control (13)	Diabetic (11)	Insulin treated diabetic (9)
Total phospholipid (TPL) (µg / mg protein)	422.35±20.95	761.84±50.34 a	683.02±31.25 a
Cholesterol (CHL) (µg / mg protein)	203.79±10.26	312.52±21.38 a	242.28±22.78 #
TPL / CHL ratio	0.98± 0.03	1.24± 0.05 a	1.38± 0.08 a

Results are expressed as mean±S.E.M. of the number of independent observations indicated in the parentheses.

a, $p<0.001$ compared with corresponding control.

#, $p<0.05$ compared with corresponding diabetic.

Table 6. Phospholipid composition of erythrocyte membranes from male rats.

	Phospholipid Composition (% of total)		
	Control (14)	Diabetic (16)	Insulin treated diabetic(11)
LYSO	2.01±0.28	1.72±0.33	2.90±0.67 b,#
SPM	10.23±1.30	7.29±0.76 c	14.25±0.93 #
PC	44.18±1.98	57.32±1.65 c	42.95±0.96 a,@
PI	8.78±0.99	2.81±0.69	5.92±0.66
PS	5.68±0.88	5.50±0.74	5.08±0.62
PE	25.31±1.31	21.93±0.98 a	23.33±1.60
PA	3.76±0.45	3.17±0.50	3.39±0.37

Results are expressed as mean±S.E.M. of the number of independent observations indicated in the parentheses.

a, $p<0.05$; b, $p<0.02$ and c, $p<0.001$ compared with corresponding control.

@, $p<0.005$ and #, $p<0.001$ compared with corresponding diabetic.

phosphatidylinositol (PI), phosphatidylserine (PS) and phosphatidic acid (PA) were the minor components (2.01, 10.23, 8.78, 5.68 and 3.76 % respectively). In diabetic group PC content increased by 30 % but the PE content decreased by 13 %. However, the major decrease was seen in PI content which amounted to 68 %. After insulin treatment SPM content increased by 39 % and 95 % as compared to the control and diabetics respectively. PC content decreased by 25 % as compared to the diabetic rats and became comparable with the control group. PI content doubled as compared to the diabetic group but was still 33 % lower than the controls.

The phospholipid composition of the erythrocyte membrane from the females in control group (Table 7) was somewhat different compared to the males in that the Lyso and PA content were higher and PI content was about half (eg. see Table 6). The PC and PE were the major phospholipids (44.22 and 26.19 % respectively). In diabetic female rats Lyso, PI and PS decreased by 79, 90 and 89 % respectively; the PC content increased by 37 %. After insulin treatment, Lyso content increased by 3.6 fold as compared to the diabetic female rats but was somewhat lower (26 %) as compared to the controls. SPM content increased by 31 % and 58 % as compared to the control and diabetic group respectively. After insulin treatment PC content decreased by 40 % as compared to the diabetic females, and remained 18 % lower as compared with the control group. Both the differences were statistically significant. In the insulin treated diabetic female rats PI

Table 7. Phospholipid composition of erythrocyte membranes from female rats.

	Phospholipid Composition (% of total)		
	Control (16)	Diabetic (14)	Insulin treated diabetic(12)
LYSO	4.62±0.33	0.95±0.21 c	3.40±0.94 @
SPM	7.80±0.70	6.47±0.71	10.22±0.56 a,#
PC	44.22±1.33	60.66±0.91 c	36.24±1.21 c,#
PI	4.94±0.62	0.48±0.24 c	9.63±1.13 b,#
PS	4.80±0.71	0.82±0.28 c	5.10±1.04 #
PE	26.19±1.44	23.69±0.53	28.86±1.30 @
PA	7.31±0.76	6.94±0.40	6.51±0.70

Results are expressed as mean±S.E.M. of the number of independent observations indicated in the parentheses.

a, $p<0.02$; b, $p<0.002$ and c, $p<0.001$ compared with corresponding control.

@, $p<0.002$ and #, $p<0.001$ compared with corresponding diabetic.

content increased by 20 % as compared to the diabetic female rats and were almost double as compared to the control female rats. PS content increased after insulin treatment and was 10 % and 22 % higher than the control and diabetic females respectively. PA value was comparable in all three groups of animals.

Table 8 shows data on fluidity of erythrocyte membranes from male rats. Fluorescence polarization (P) value increased significantly in the diabetic male rat group as compared to the control males. Marginal decrease in P value and for fluorescence anisotropy (r), limited hindered anisotropy (r_∞) and order parameter (S) was observed after insulin treatment.

Table 9 shows data on fluidity of erythrocyte membranes from female rats. Fluorescence polarization (P) value decreased significantly in the diabetic female rats as compared to the control female rats, which was opposite to that seen for the male rats (Table 8). In insulin treated diabetic female rats, the P value increased significantly beyond the control value. The same trend was observed for fluorescence anisotropy (r), limited hindered anisotropy (r_∞) and order parameter (S).

Discussion

The present experiments were initiated to find out if sex-dependent differences are evident in the basic physiology and

Table 8. Fluidity of erythrocyte membranes from male rats.

Fluorescence parameter	Control (22)	Diabetic (18)	Insulin treated diabetic(17)
Fluorescence polarization(P)	0.271±0.004	0.542±0.044[a]	0.451±0.018[a]
Fluorescence anisotropy(r)	0.199±0.003	0.454±0.044[a]	0.356±0.017[a,@]
Limited hindered anisotropy(roc)	0.165±0.004	0.511±0.058[a]	0.374±0.022[a,@]
Order parameters(s)	0.909±0.003	1.035±0.012[a]	1.019±0.008[a]

Results are expressed as mean±S.E.M. of the number of independent observations indicated in the parentheses.

a, $p<0.001$ compared with corresponding control.

#, $p<0.05$ compared with corresponding diabetic.

Table 9. Fluidity of erythrocyte membranes from female rats.

Fluorescence parameters	Control (24)	Diabetic (6)	Insulin treated diabetic(14)
Fluorescence polarization(P)	0.286±0.003	0.158±0.013 b	0.349±0.017 b,#
Fluorescence anisotropy(r)	0.211±0.026	0.111±0.010 a	0.264±0.015 #
Limited hindered anisotropy(roc)	0.181±0.004	0.048±0.013 b	0.252±0.019 a,#
Order parameters(s)	0.926±0.003	0.633±0.062 b	0.968±0.012 a,#

Results are expressed as mean±S.E.M. of the number of independent observations indicated in the parentheses.

a, $p<0.002$ and b, $p<0.001$ compared with corresponding control.

#, $p<0.001$ compared with corresponding diabetic.

the erythrocyte membranes from the alloxan-diabetic rats. The rationale for these studies was that the incidence of CHD and CHF is higher in human female diabetics than in the males (38).

Loss of body weight in experimental animals in diabetes is well established and our observations (Table 1 and 2) match with the reports of other researcher (39-41). The extent of weight loss was about the same in both male and female rats. However, the increase in the blood sugar level was much higher in the diabetic females than in the males. By contrast, the urinary sugar levels and daily urinary sugar excretion was higher in the diabetic males than in the diabetic females (Table 1 and 2). Effects of insulin treatment were also differential. The restoration of body weight was much less in the females than in the males. Likewise the control of blood sugar level was also much less efficient in the females.

With respect to erythrocyte membrane and serum protein glycosylation also sex-dependent changes were noted. Extent of erythrocyte membrane protein glycosylation was much high in the males and diabetic state had no effect; in the females the diabetic state significantly enhanced erythrocyte membrane protein glycosylation. This may be related to the high blood sugar levels noted above (Table 2). The picture for the serum protein glycosylation was opposite to that of

erythrocyte membrane glycosylation.

Our value for erythrocyte membrane TPL and CHL match with the earlier reported values (20). Especially the decreased CHL content is noteworthy (20). Decreased CHL content together with decreased cholesterol/phospholipid ratio in streptozotocin diabetic rats has been reported (42). The extent of increase in the TPL was of lesser magnitude in the diabetic females and the CHL content increased by 50 %. Insulin treatment controlled TPL content to some extent in both male and females but was effective only in the males in controlling the CHL content. The results thus emphasize the sex dependent differential effects. In this connection it is interesting to note that the RBC membranes acquire their CHL by process of exchange from the plasma (20).The results thus imply that the process of exchange may be differentially affected in the males and females by diabetic state.

Sex-dependent differences were observed once again even in the control group for the erythrocyte membrane phospholipid composition (Tables 6 and 7). Thus in the membranes from the females the proportion of acidic phospholipids PI and PS was about half compared to the males. Diabetic state resulted in decreased PE and acidic phospholipid contents in the males. In the females the decrease in the acidic phospholipid i.e. PI and PS was of substantially greater magnitude (Table 7). Insulin treatment had PC lowering effect in both the sexes and was able to restore levels of acidic phospholipids. PE

content was restored effectively only in the females after insulin treatment.

In diabetic male rats the erythrocyte membranes became more rigid while in the females the membrane was fluidized, which once again points to the basic difference between the two sexes. Insulin treatment was more efficacious in the females than in the males in restoring membrane fluidity (Tables 8 and 9).

Reports on effect of diabetes on erythrocyte and platelet fluidity are controversial (21-25). However, the present studies have demonstrated a clear-cut decrease in the membrane fluidity in the experimental animal model of diabetes. It was tried to reason out why the effects were differential for males and females. For this attempts were made to correlate the membrane fluidity with PC/PE, SPM/PE and TPL/CHL ratios (33, 43). It was observed (data not shown) that in the males the fluorescence polarization P correlated positively with PC/PE and TPL/CHL ratios. By contrast in the females positive correlation with SPM/PE and a strong negative correlation with TPL/CHL ratios was evident.

Since the membranes from the females accumulated less amount of TPL and insulin treatment had CHL lowering effect, it would seem that the viscosity and the rheological properties of the erythrocytes membranes from the females may be

affected less under these conditions. Therefore defect in the RBC membrane may not be a primary cause of increased incidence of CHD and CHF noted for the diabetic females (38).

Summary

Effect of alloxan induced diabetes and insulin treatment on erythrocyte membranes and serum in male and female rats were examined.

The blood sugar level increased to a greater extent in the diabetic female rats than in the male rats. However, the urinary sugar levels and daily urinary sugar excretion was higher in the diabetic males.

The diabetic animals lost considerable body weight; the restoration of body weight and control of blood sugar level was less efficient in the insulin treated diabetic females.

Sex dependent changes were noted in erythrocyte membrane and serum protein glycosylation.

The extent of increase in the TPL in the erythrocyte membranes was of lesser magnitude in the diabetic females and the CHL content increased by 50 %. Insulin treatment controlled TPL content to some extent in both male and females but was effective only in the males in controlling the CHL content.

Diabetic state resulted in decreased acidic phospholipid content in the erythrocyte membranes from the males. In the females the decrease in the acidic phospholipid was of

greater magnitude. Insulin treatment was able to restore levels of acidic phospholipids. In diabetic males the erythrocyte membrane became more rigid while in the females the membrane was fluidized.

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