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# Hyperglycemia Induced Osmotic Disequilibrium, Oxidative Stress and Cell Death in Type II Diabetes Patients.

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**ABSTRACT**

High unutilized glucose in the body in diabetes along with increased level of Na+ and urea cause altered osmotic equilibrium in the vascular bed contributing altered cellular microenvironment which may further affect membrane function. As plasma membrane is a dynamic structure, it is responsive to chemical exposure. Any chemical exposure may trigger alteration in membrane function and subsequently down-hill reactions. We are therefore studying the role of high glucose concentration in membrane alteration, altered cellular function and cell death in diabetic patients. In our present study we have recruited newly diagnosed 30 type II diabetes mellitus patients, chosen from diabetic clinic, Howrah Zila Hospital, Howrah, West Bengal and 31 age and sex matched healthy control without any history of diabetes from same socio economic background. Significantly high glycemic index (studied by serum glucose level and glycated haemoglobin level), high blood osmolality, osmotic fragility and oxidative stress have been noted in every diabetic participant in comparison to non-diabetic individual of our study population. A significant cell membrane alteration ($p<0.05$) with altered serum free sialic acid concentration has been noted in diabetic patients. Degree of membrane deformity, frequency of nonviable WBC, frequency of apoptotic cells and length of comet tail all are significantly higher ($p<0.01$) in diabetic persons in comparison to normal non-diabetic counterpart of the population. Diabetes is an altered homeostatic state which contributes to cellular dis-integrity and cell death and is associated with osmotic dis-equilibrium.

**Keywords:** Diabetes, cell membrane, cell death, Osmotic equilibrium, oxidative stress.

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## INTRODUCTION

Diabetes mellitus is characterized by absolute or relative deficiencies in the serum level of insulin. About 6% of our total world population is affected by diabetes in 2007 and the ratio will increase upto 7.3% by 2025. Globally, type II diabetes mellitus is the 3rd and 10th leading cause of mortality and morbidity among women and men [1,2] . Diabetes mellitus can be characterized by metabolic disorders in which a person has high blood sugar, dyslipidemia with altered BMI. This high blood sugar produces the classical symptoms of polyuria, polydipsia and polyphagia [3]. Loss of adequate insulin secretion or insulin deficiency to the target site promotes significant hyperglycemia with disturbances of carbohydrate, lipid and protein metabolism [4, 5]. These metabolic dysfunctions alter the cellular microenvironment of the vascular bed of the body to cause myriad clinical manifestations with significant musculo-skeletal weakness and anaemia. Diabetes mellitus, a metabolic disorder, characterized by hyperglycemia, is associated with long term complications including retinopathy, nephropathy , neuropathy and angiopathy [1-5].

Hyperglycemia is considered as a primary cause of diabetic vascular complications and is associated with oxidative stress, impaired trace element and lipid metabolism as well as pancreatic enzyme abnormalities [6] . High glycemic index, significant oxidative alteration/ maladjustment may act as the promoting factors for vascular abnormalities in diabetes and may in turn cause alteration of cellular microenvironment and homeostasis.

Otton and associates [7,8] reported that, diabetes produces significant alteration in morphology and half life of lymphocytes with altered metabolism. Diabetes associated cell death of pancreatic beta cells and neutrophil [9, 10] and in a separate study of testicular cell [11] has also been reported. So, we are hypothesizing that, like lymphocytes other cellular compartments of blood are also the target of diabetes for induced cell death.

Diabetes is associated with significant defects in antioxidant defence mechanism and generation of reactive oxygen species (oxidative stress) which may play an important role in the etiology of diabetic complications. Several antioxidant system of the body shows significant alterations [12]. Decrease of superoxide dismutase (SOD), catalase (CAT), peroxidise (Px), ceruloplasmin (Cp) and glutathione peroxidise (GSH-Px) activities as well as a decrease in the GSH level and an increase in the concentration of glutathione disulfide (GSSG) were observed in erythrocytes and other tissues of diabetic patients [13 , 14] .

Diabetes is a state of altered osmotic equilibrium because of the increased glycemic index in plasma. The high levels of glucose and $Na^+$ inside the blood vessels create osmotic disequilibrium and alter the cellular microenvironment in blood vessels and tissue space.

We are hypothesizing that, as diabetes is a state of altered osmolality due to increase of unutilised glucose molecules inside the blood vessels in association with Na and urea, it may induce cell death.

We are therefore, trying to investigate the changes in cell morphology and frequency of cell death in diabetes. The present study is also trying to estimate the oxidative damage (if any) in the cellular compartment of blood to get an idea whether ROS and osmotic disequilibrium may contribute to cellular death or not.

## MATERIALS AND METHOD

### Subject selection

30 type II diabetic patients were recruited from Howrah Zila Hospital. Diabetes was confirmed by assaying serum fasting glucose level and level of glycated haemoglobin. All of the patients were suffering from diabetes from unknown time period and they were diagnosed with diabetes for the first time and they were not under medication before then. Clinical examinations for other systemic manifestations were judged by expert clinicians of the hospital and confirmed cases were selected for the study. Inclusion criteria for the study were not suffering from any other hormonal or infectious disease and not under in any type of antibiotic medication. 31 age and sex matched non-diabetic persons were also chosen for the study to serve as control. 2.5 ml of EDTA anticoagulated blood and 2.5 ml of whole blood samples were collected from cases and

control subjects of the study population by veni-puncture after taking written informed consent from all of them. Ethical guideline was followed at every step of the work formulated by Medical Council of India, according to Helsinski rule. Ethical clearance was taken from Institutional Human Ethical Committee, Presidency College. All of the serum and whole blood samples were transported into an ice bag to the Environmental Toxicology Research laboratory, Presidency University (formerly Presidency College) where the blood and serum samples were kept in -20 ◦ C until further analysis.

**Morphological study with light microscope**

Blood smear was prepared on glass slide and stained with Leishman's stain (contains methylene blue and eosine dissolved in methyl alcohol). After preparing the slides were viewed under microscope for morphological change and comparison was made with control. Number of cells with membrane alteration in both cases and control in was calculated in four different fields under the microscope.

**Study of morphology with Scanning Electron microscopy**

Packed cell were prepared by centrifugation of the fresh anticoagulated whole blood samples and WBC were separated by ficol-Histopaque. Packed cell and WBC only cell population then washed with PBS. The cells were then fixed with glutaraldehide and transported in ice bucket to Jadavpur University, Instrumentation centre for central instrumentation facility. The SEM pictures were taken there.

**Biochemical analysis**

**Serum Glucose and Glycated Haemoglobin**

Serum glucose level was assayed by GOD-POD method (End Point) in which the ratio of absorbance of standard and unknown was multiplied by concentration of standard in mg/dl (product code/catalogue No: KGGLU 104.1.1).

Glycated hemoglobin was estimated by cation exchange resin method. In this method hemolysate is prepared by mixing venous blood with lysing reagent. Labile Schiff base is eliminated during hemolysis. The hemolysate is then mixed with a weakly binding cation exchange resin. The nonglycosylated hemoglobin binds to the resin leaving GHb free in the supernatant. The GHb percentage is determined by measuring the absorbance of GHb fraction and the total Hb at 415nm in semiautoanalyser [15] .

**Serum Na and K**

Serum Na, K, urea and creatinine were estimated by Na, K estimation Combi kit and urea and creatinine estimation kit from mserum, manufactured and marketed by Span Diagnostics,(Autospan liquide gold creatinine and urea) Surat, india.

**Serum free Sialic acid**

Serum free sialic acid was estimated from every samples by periodic acid and resorcinol method. N-acetylneuraminic acid (Cat. No. A-9646) was purchased from Sigma Chemical Co., MO, USA. 0.5 ml of blood was mixed with 0.1ml of 0.04 M periodic acid and thoroughly mixed and kept for 20 min in ice bath. 1.25 ml of resorsinol reagent was added and mixed well, then placed in an ice bath for 5 min and subsequently heated at 100º C for 15 min. After cooling in tap water 1.25 ml of tertiary butyl alcohol was added. After vigorous mixing, it was placed in 37º C water bath for 3 min to stabilize the colour. The colour intensity was measured at 630 nm [16] .

**Blood Osmolality and Osmotic fragility**

Serum osmolality (miliosmole/lit) was estimated in every samples by the following formula

Serum osmolality= 2(Na+K) + (glucose/18)+ (urea/2.8) [17] .

Osmotic fragility of blood cell was estimated by simple colorimetric method. Graded concentration (0.9%, 0.83%, 0.7%, 0.64%, 0.53%, 0.47% and 0.38% ) of NaCl in PBS was prepared. For each sample, 2.5 ml of each 7 different concentration of NaCl in PBS was aliquot in 7 test tubes and 50 µl of fresh blood sample was added to each of the tube and allowed to stand for 30 min at room temperature after thorough mixing. The tubes were centrifuged at 2000 rpm for 5 min and supernatant was collected. The OD was then taken at 540 nm which gives the account of relative haemolysis in RBC.

**Determination of Lipid peroxidation**

MDA was measured as an index of lipid peroxidation [18]. Briefly, plasma samples (0.3 ml) were mixed with a trichloroacetic acid/thiobarbituric acid/HCl reaction solution (5:2:1, by vol.), heated for 60 min in a boiling water bath, cooled with tap water and centrifuged.

The absorbance of the supernatant was recorded spectrophotometrically at 532 nm with 1,1,3,3-tetraethoxypropane as a standard (results are expressed as µmol of MDA formed/l of plasma).

**MTT assay**

MTT assay of cell viability was performed according to the method described earlier [19] . Whole fresh blood was treated with ficol-Histopaque to isolate the buffy coat. White blood cell population was isolated and washed in PBS to make a final cell suspension of 600 µl. The suspension was subdivided into 5 microcentrifuge tubes. Among these 5 tubes 4 was incubated at 37 ˚C with dexamethasone (10µM) to a final volume of 200µl for 0,1,2,3 hrs respectively with a control having no dexamethasone. In each of the cell suspension 20µl of MTT (5 mg/ml in PBS) was added and incubated further for another 3 hrs. The mixture was then centrifuged at 5000 rpm for 5 min and the pellet was re suspended in 100µl of DMSO and optical density was taken at 540nm. With each of the diabetic sample one or more control was assayed similarly to compare with the control.

**Cell viability assay by Tryphan Blue**

Single cell suspension was made from fresh blood by ficol paque in PBS and equal volume of 0.4% tryphan blue (in PBS) was added. Suspension of cells was observed under the microscope to differentiate between live and dead cells. Cells with blue cytoplasm (dead) and cells with transparent or unstained cytoplasm (live) was viewed and the percentage of live cell was calculated by a haemocytometer under the microscope [20] .

**Assay for apoptosis**

100 µl of whole blood was diluted with PBS (1:1) and incubated at 37º C for 10 mins. Cells were separated by centrifugation at 5000 rpm and suspended in 50 µl of PBS. Three aliquot of cell suspension were prepared from each and 10 µm of dexamethasone was added in each aliquot and incubated further for 1, 2 and 3 hours. After completion of the incubation blood film was prepared and stained with acreydine orange (AO) and propidium iodide (PI). 10 µl each of AO (AO; Invitrogen, USA) and PI (PI; Invitrogen, USA) ( 20 µg/ml each, in PBS for 5 to 10 min without any fixation) was used for staining and observed under a fluorescence microscope (Axiolab fluorescence microscope ,Zeiss, Germany) [21] using blue filter and compared with control. Study of apoptosis was also done in the same manner without adding dexamethasone in diabetes and in control blood cell to compare the effect of dexamethasone on cell death in diabetic and control blood.

**Assay of COMET**

Single cell suspension was made from fresh blood using ficol and count was taken. Cells, after mixed with low melting agarose, were placed over frosted slide to immobilize them. An additional layer of agarose was then applied over it and the slide was placed within alkaline lysis solution. It was then electrophoreses and stained with Ethidium Bromide and then viewed under fluorescence microscope [22] .

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**Study of DNA laddering**

DNA was isolated from control and diabetic peripheral blood leucocytes by conventional chloroform extraction method [23] and electrophoresd with 100 bp DNA ladder in a 1% agarose gel for 1 hr in 70 mv.

## RESULTS

Blood cells of diabetic persons showed a membrane alteration in both RBC and WBC. Corrugation of plasma membrane in RBC and WBC is quite prominent (fig.1a,1b). The percentage of cells having membrane corrugation is 47% in diabetic persons on an average while the percentage of such cells is only 15% ($p<0.05$) in case of normal non diabetic person in circulation.

**Table 1: Demographic distribution of study population.**

| Characteristics | Control (n= 31) | Diabetic (n=30) |
|---|---|---|
| Age (years) | 40.33 ± 6.44 | 47.15 ± 7.87 |
| Sex<br>Male<br>Female | <br>N=26<br>N=5 | <br>N=21<br>N=9 |
| BMI (kg/m2) | 24.66 ± 5.76 | 26.43 ± 7.32 |
| Total calorie intake (Cal) | 2300 ± 680 | 2400 ± 724 |
| Smoking habit | Smoker (n) = 26<br>Non-smoker (n)= 15 | Smoker (n) = 9<br>Non-smoker (n)= 21 |
| BP systolic (mmHg)<br>BP diastolic (mmHg) | 116 ± 14<br>74 ± 8 | 122 ± 11<br>79 ± 12 |

All of the subjects included in the study population are moderately active sedentary worker.

**Table 2 :- Comparison of biolochemical parameters between control and diabetic persons of study population.**

| GROUP | GLUCOSE (mg/dl) | GLYCATED Hb (%) | $Na^+$ (mEq/dl) | $K^+$ (mEq/dl) | UREA (mg/dl) | CREATININE (mg/dl) | SERUM PROTEIN(OD at 660 nm) |
|---|---|---|---|---|---|---|---|
| CONTROL | 83 ± 1.05 | 3.5 ± 0.1 | 135 ± 2.13 | 3.5 ± 0.33 | 15 ± 0.5 | 0.7 ± 0.1 | 0.266 ± 0.07 |
| CASE | 130 ± 2.02 | 7.4 ± 0.07 | 142 ± 2.5 | 4.5 ± 0.4 | 22 ± 0.89 | 1.14 ± 0.2 | 1.84 ± 0.04 |
| SIGNIFICANCE VALUE | P<0.05 | P<0.05 | P<0.01 | p>0.001 | p>0.001 | P<0.01 | p>0.001 |

**Table 3: Representation and comparison of osmotic fragility of RBC in graded concentration of NaCl between control and Diabetic person.**

| GROUP | 0.9% NaCl | 0.83% NaCl | 0.7% NaCl | 0.64% NaCl | 0.53% NaCl | 0.47% NaCl | 0.38% NaCl |
|---|---|---|---|---|---|---|---|
| CONTROL | 0.54 ± 0.06 | 0.645 ± 0.2 | 0.68 ± 0.09 | 0.77 ± 0.04 | 0.9 ± 0.45 | 0.95 ± 0.05 | 1.1 ± 0.14 |
| CASE | 0.66 ± 0.2 | 0.69 ± 0.1 | 0.71 ± 0.19 | 0.8 ± 0.12 | 0.794 ± 0.1 | 0.86 ± 0.28 | 0.93 ± 0.39 |
| SIGNIFICANCE LEVEL | p>0.05 | p>0.05 | p>0.05 | p>0.05 | p>0.05 | p>0.05 | p>0.05 |

**Table 4: Oxidative stress count in diabetes and comparison with control subjects.**

| | Normal | Diabetic | Level of Significance |
|---|---|---|---|
| Serum MDA level (µmol/l) | 0.87 ± 0.29 | 4.07 ± 0.71 | P<0.001 |
| Serum Vit C level (µmol/l) | 47.33 ± 8.11 | 28.07 ± 9.13 | P<0.001 |
| GSH-Px (u/gHb) | 62.21 ± 13.91 | 70.56 ± 14.77 | P<0.001 |
| SOD (u/gHb) | 1499.63 ± 339.44 | 1571.07 ± 256.77 | P<0.001 |

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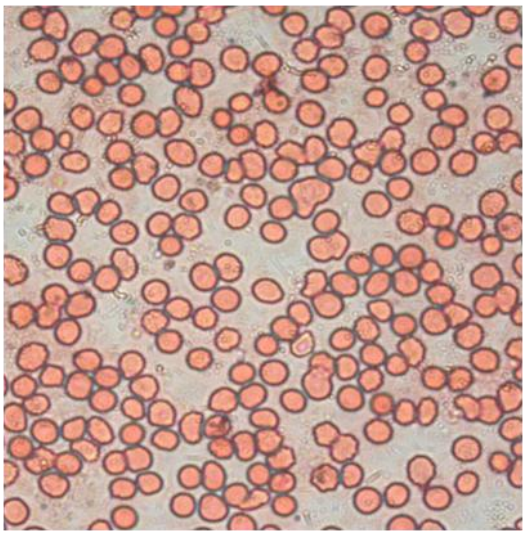
**Table 5 : Mean values of MTT assay, Tryphan blue exclusion assay, comet assay and comparison between control & Diabetic study population**

| Characteristics | Control | Diabetic | P value |
|---|---|---|---|
| Tryphan blue exclusion assay (% of live cell) | 75. 61± 11.34 | 51± 9.87 | P<0.05 |
| MTT assay | 0.113 ± 0.061 | 0.169 ± 0.01 | <0.025 |
| Comet assay % of apoptotic cell | 17.54 ± 4.51 | 31.66 ± 6.32 | p<0.01 |
| % of tail DNA | 1.39 ± 0.67 | 3.54 ± 1.03 | p<0.05 |
| Olive tail moment | 0.67 ± 0.14 | 2.04 ± 0.51 | p<0.05 |
| Sailic acid (g/l) | 0.67 ± 0.06 | 0.85 ± 0.04 | P<0.05 |
| Osmolality (mosm) | 294.25 ± 4.32 | 330.74 ± 9.68 | p<0.05 |

**Figure 1: RBC memebrane alteration in diabetes (b) in comparison to normal non diabetic RBC**



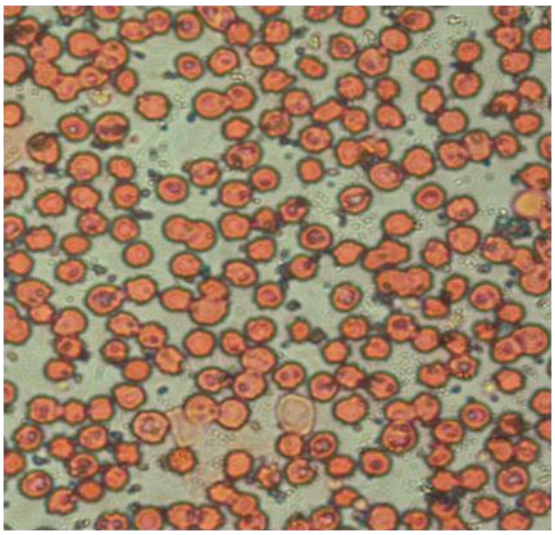
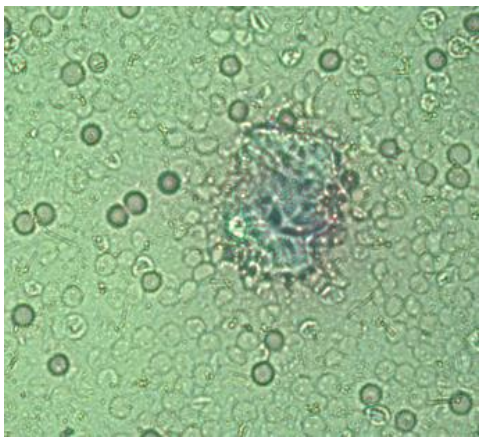


**Fig 1a (control)** **Fig 1b (diabetes)**

**Figure 2 : Clumping of cells in diabetes.**



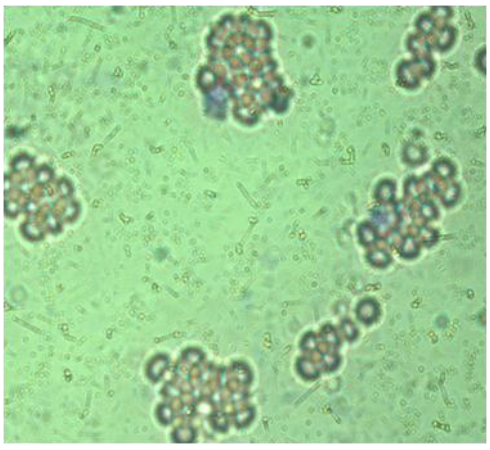


**Fig 2a (control)** **Fig 2b (diabetes)**

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**Figure 3: Tryphan blue exclusion assay for checking cell viability. The live cells not take stain and only the dead cells take stain.**

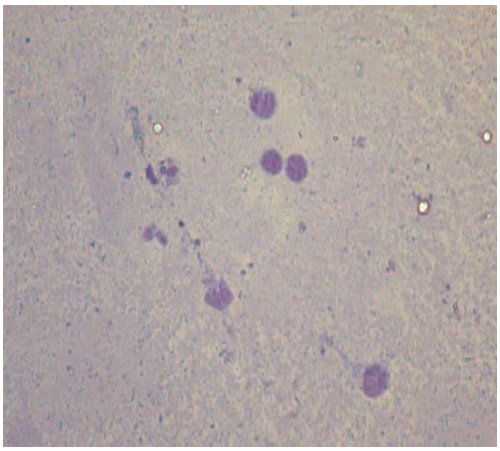

**Figure 4: Comparative study of RBC morphology by SEM in control and Diabetic patients.**

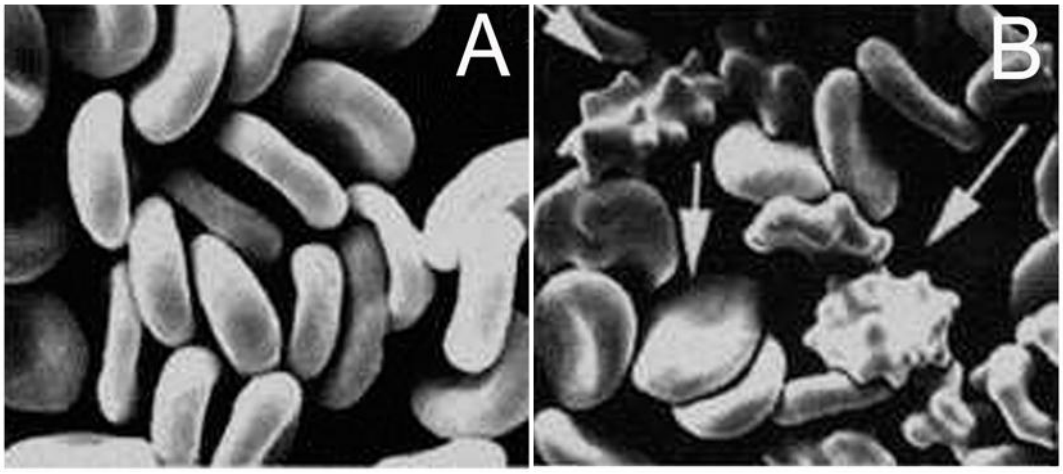


**Fig 4 a (control)** **Fig 4b (diabetes)**

**Figure 5: Comparative assay of apoptosis in peripheral blood cells of control and diabetic persons.**

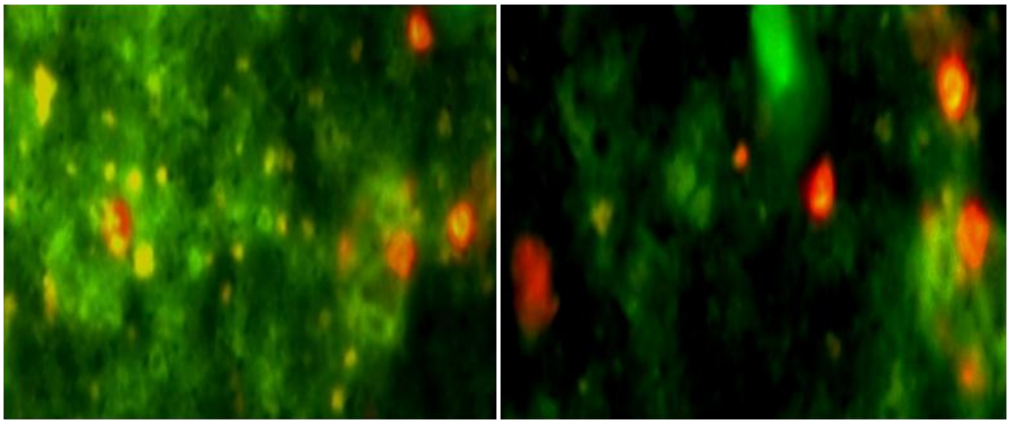

**Fig 5a (Control)** **Fig 5b (Diabetic)**

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**Figure 6: Comparative analysis of Apoptosis in normal (cell death induced by Dexamethasone) and in Diabetes ( with out dexamethasone in diabetes)**

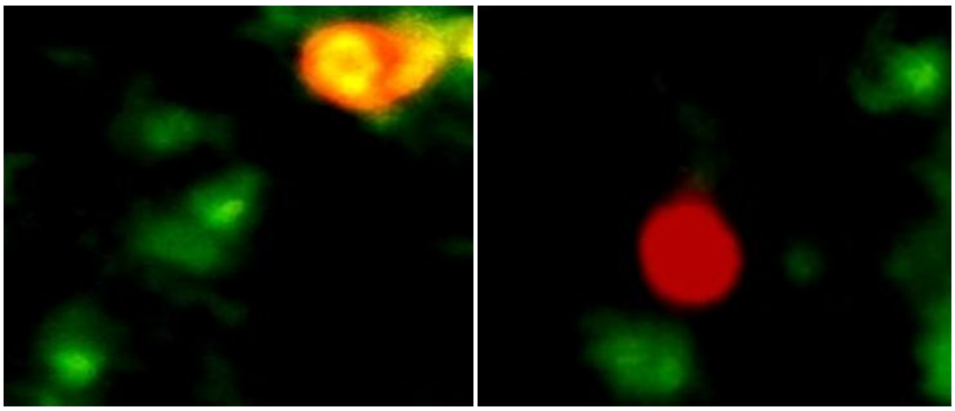


**Fig 6a (Control)** **Fig 6b (diabetes)**

**Figure 7: Assay of COMET in diabetic peripheral blood leukocyte .**

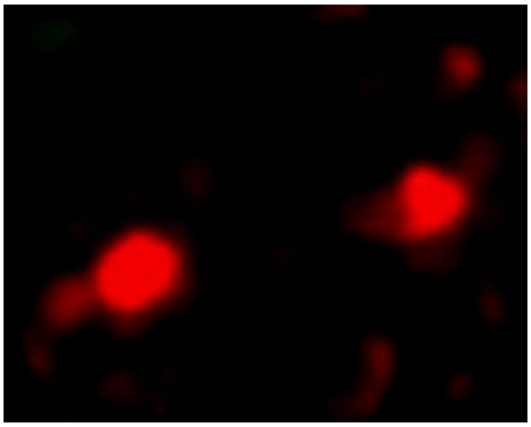
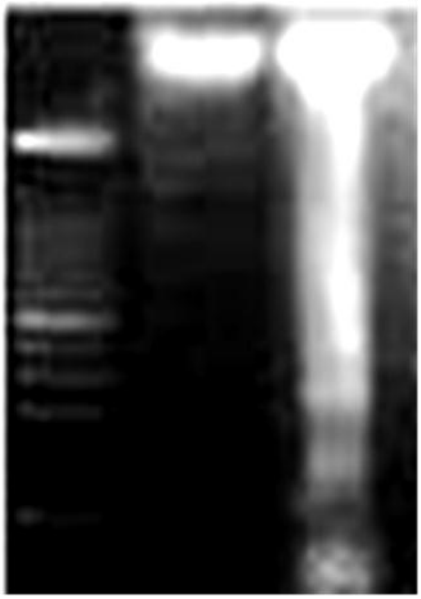


**Figure 8 : Agarose gel electrophoresis for study of DNA degradation**

Lane 1 Lane 2 Lane 3



Lane1: MW , Lane 2: Control, Lane 3: Case

Cell taken from diabetic persons showed a tendency to form clump in comparison to non diabetic persons (fig 2a, 2b). Blood taken from diabetic persons showed a significantly higher level of serum free sialic acid ($p<0.05$) in comparison to normal counterpart of the subject population. Serum level of glycated haemoglobin, Na+, K+, urea, creatinine and serum total protein are significantly altered ($p<0.05$, $p<0.001$); (table 1) in diabetic persons in comparison to normal persons. Plasma osmolality and osmotic fragility of red blood cell are significantly different in diabetic persons in comparison to normal counterpart of the population. We have studied reduced glutathione, superoxide dismutase , MDA, vitamin C in both the groups indicating a significant oxidative damage in diabetic persons. RBC from diabetic persons has shown lower level of osmotic fragility after placing in hypotonic solution. In case of diabetic persons a significant decrease in osmotic fragility ($p<0.05$) and significant increase in plasma osmolality ($p<0.05$) has been notified (table2). We have shown a significant membrane blebing in cells taken from diabetic persons in comparison to normal one which had been reflected by SEM study (Fig 3a, 3b). The cells of diabetic persons were checked for viability by tryphan blue exclusion assay (fig 4) and MTT assay. Both the assay signified that cells (lymphocytes) taken from diabetic origin have a shorter half life than from normal non diabetic cells. The percentage of live cells were significantly less in diabetes in comparison to non diabetic persons ($p<0.05$). We have found a significantly higher rate of apoptotic cells, studied by acrydine orange and propidium iodide staining, in diabetes when compared with non-diabetic counterpart (fig 5). Apoptosis, studied in diabetes and non-diabetes persons, also showed significant association with diabetes when the samples were pre-treated with dexamethasone, an agent induces apoptosis (fig 6). COMET assay also showed significant association with DNA of diabetic persons isolated from peripheral blood leucocytes (fig 7). Electrophoresis of DNA isolated from peripheral blood leukocytes of both control and diabetic individual has shown significant DNA fragmentation in case of diabetic DNA in comparison to control (Fig 8). This DNA laddering also indicates significant degree of DNA degradation and cell death in diabetes.

## DISCUSSION

Hyperglycemia, characterized by increased blood glucose level, increases the concentration of osmotically active particles in the blood vessels and create hyperosmolality of blood.

Altered osmotic equilibrium is said to be involved in altered cellular functions including $Na^+$ and $K^+$ conductance of the cells placed in this changed microenvironment. Our results of osmotic fragility tests and $Na^+$, $K^+$ concentration of blood taken from diabetic patients, also support this (Table 1).

Alteration of diabetes induced osmotic equilibrium and changed membrane conductance has also been reported on isolated Glial cells of rat retina [24]. Hyperosmotic stress induced corneal cell death unrelated to diabetes has been reported later on [25, 26].

Apoptosis is a controlled and regulated process of cell death. Apoptosis and cell proliferation are in balance in healthy organisms. In situations like alteration in homeostasis this balance may be lost and may cause unregulated cell death [27, 26]. We hypothesized that osmotic disequilibrium or oxidative stress may cause alteration in cellular homeostasis and thereby may promote apoptotic death. Hyperosmotic stress and glyco-oxidation are known factors for altering cellular functions and a source of oxidative damage and DNA damage. Hyperglycemic state glycosylates hemoglobin, creates oxidative stress and puts the cellular components at risk. Therefore in diabetes induced hyper osmotic state, RBC membrane composition has been analyzed and we have found an altered level of serum free sialic acid which is thought to be a basic component for maintenance of cell membrane in its normal shape and size. Increased level of free sialic acid in serum support our finding, and we have also found that diabetes has an increased number of dying cell with irregular shape and size (altered morphology) in the general circulation. Association of increased glycemic index in diabetic patients with altered cellular morphology, decreased cellular viability and increased DNA degradation has been noticed earlier in two separate study [28,29]. In our study a significantly increased percentage of cells with altered morphology with increased number of dead cell has been noticed by tryphan blue exclusion assay, MTT assay and study of apoptosis. Electrophoresis of genomic DNA has been showed DNA laddering and partial smearing in 1% agarose gel.

It has also been reported earlier that diabetes is associated with increased oxidative damage in cell [30]. Our findings also support the same (table 3) with a lower level of reduced glutathione, Super Oxide Dismutase, Catalase, Vitamin C and higher level of MDA in diabetic patients in comparison to normal

counterpart. This result signifies that oxidative stress associated with diabetes may promote the membrane alteration and cellular damage in this group of patients. Increased cellular load of glucose is thought to inhibit pentose phosphate pathway which is a key source of NADP necessary to restore the reduced form of glutathione, one of the most important antioxidant defence. High cellular glucose levels are also responsible for inhibition of the enzyme glucose 6 phosphate dehydrogenase involved in the pentose phosphate pathway [31]. This enzyme inhibition may be a crucial factor for fewer amounts of energy generation and resultant weakness in diabetes. Although acute glucose load to the astroglial cell showed increased pentose phosphate pathway activity [32].

Change of cellular osmolality has been contributed in our study subjects by alteration of plasma glucose level. Along with plasma glucose level plasma Na, K urea and creatinine concentration is the maintaining factor for change of plasma and cellular osmolality in diabetes. Change of ionic concentration in diabetes increases the plasma osmolality in our subjects to 340-350 mosm from the normal range of 270-300 mosm. This change of osmolality actually affects transmembrane ionic gradient of blood cells and thereby provoke functional alterations in ionic transporters. Perturbation of cell volume and cell shrinkage has been reported to be associated with factors, like Na+ and K+ imbalance in extracellular as well as intracellular fluid and increased glycemic index of blood [33]. According to the "pump and leak" concept, cell swelling and lysis can be avoided by a combination of a low Na+ permeability and active Na+ extrusion via the Na –K -ATPase which increases extracellular fluid Na concentration. Again altered concentration of Na+ renders the plasma membrane effectively impermeable to Na + for blood cell [33] . This leads to altered cellular homeostasis and functional impairment. Cells in anisotonic solution usually regulate cell volume and morphological equilibrium either by regulatory volume decrease (RVD) or by increasing cell volume towards the original value, by the process of regulatory volume increase (RVI). RVD is dependent on increases in the net efflux of Cl-, K+, and organic osmolytes, whereas RVI involves the activation of Na+ cotransport, Na/H exchange, and nonselective cation channels. Under normal physiological conditions, the osmolality of the extracellular fluid is kept constant by body fluid homeostasis (285 mosmol/kgHO), and cell volume is most commonly perturbed by changes in intracellular or extracellular osmolality [33] .

Increased proportion of apoptotic lymphocytes has been noticed in general circulation of diabetic patients [7]. We have also found considerable fraction of dead lymphocyte in the peripheral blood smear taken from diabetic patients in comparison to normal counter part of the population.

However, change of cellular or extracellular osmolality is crucial for altered osmotic pressure and thus for cellular homeostasis. Association of increased plasma osmolality (370 mosm) with an increased incident of cell death has been studied in cell culture system of chrondrocytes [34] .

Suicidal death of RBC or eryptosis has been demonstrated to be significantly associated with an increase of the extracellular K+ concentration and by functional alteration of the Ca + sensitive K+ channels [35, 36] . Cell death by apoptotic mechanism includes cell shrinkage which is partially contributed by cellular loss of K+. This cellular declination of K+ has been associated with apoptosis and [37] is triggered by increased cytosolic Ca+ activity leading to activation of Ca+ sensitive K+ channel and subsequent loss of K+ from cell to contribute to a high extracellular K+.

From the above fact we are hypothesizing that perturbation of osmotic equilibrium in the extracellular fluid contributed by increased glucose, Na and K, urea concentration in diabetes and this actually alters functional status of the plasma membrane ionic pump and therefore intracellular gradients of ions which may further alter the cellular homeostasis to contribute cell death.

RBC apoptosis imparts significant resistant to the tissue perfusion pressure of $O_2$. Significant loss of $O_2$ carrying capacity and therefore loss of tissue oxygenation causes deprivation of metabolic events in the tissue and therefore may cause depletion of oxidative phosphorylation in active cells [38]. The result is loss of ATP generation and loss of cytoplasmic pool of high energy molecules which in turn give rise to morbidity and general weakness in diabetes . On the other hand lymphocyte apoptosis plays an important role in immune function. Removal of WBC from the circulation disrupts the normal immune function. Apoptosis removes developing lymphocytes that fails to express an antigen receptor and thereby ensuring a functional repertoire of mature B and T cells and it maintains tolerance toward self by eliminating lymphocytes with antigen receptors that recognize autoantigens .

Protein glycation and protein oxidation are the major contributing factors for diabetes induced complications. Increase in blood glucose level ensures increased level of glycosylation in proteins inside the cell as well as outside to produce functional alterations [39] . Normally auto-oxidation of glucose leads to generation of ROS and reactive keto aldehydes producing free radical induced protein and lipid degradation. Increased glycation of haemoglobin (Hb) and other cellular protein as an effect of increased glucose level contribute generation of free radical [40] . Hb glycation induces release of iron and causes iron induced free radical generation. This free radical may cause biological oxidation of membrane proteins including loss of erythrocyte membrane integrity [41] .

Increased blood glucose stimulates non enzymatic glycation of proteins including albumin, crystalline, collagen LDL and Hb. The free amino group of protein causes chemical modification of proteins in presence of glucose to induce irreversible glycation of protein. In case of Hb glycation the free N terminal amino group of the B chain is linked with glucose to produce glycated Hb [42]. Advanced glycation end products (AGE) may binds with receptors on the endothelial membrane and induces expression of several chemo-attractant factors and generate ROS which then in turn may induce apoptosis [39,40,43] . Similarly, cell membrane protein having free amino terminal end can be modified and glycated. These glycation induces physical as well as chemical changes in the protein which can ultimately modify the function [37].

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