STUDY OF OXIDATIVE STRESS *PARAMETERS, GLYCATED HAEMOGLOBIN AND LIPID PROFILE IN DIABETIC RETINOPATHY*

BY

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Dissertation Submitted to

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In Partial fulfillment of the Requirements for the Degree of

M.D in **BIOCHEMISTRY**

Under the guidance of

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APRIL 2011.

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LIST OF ABBREVIATIONS

ADA	American Diabetes Association
AGE	Advanced Glycosylation End product
ANOVA	Analysis of variance
CAD	Coronary artery disease
DAG	Diacylglycerol
DCCT	Diabetes Control Complication Trial
DM	Diabetes Mellitus
DNA	Deoxyribonucleic Acid
DPN	Diabetic Peripheral Neuropathy
DR	Diabetic Retinopathy
ESR	Electron Spin Resonance
ESRD	End stage renal disease
ETDRS	Early Treatment Diabetic Retinopathy Study
FBS	Fasting blood sugar
GHb	Glycosylated hemoglobin
GOD	Glucose oxidase
GSH	Reduced Glutathione
GTT	Glucose Tolerance Test
Hb	Hemoglobin
HbA_{1c}	Hemoglobin A_{1c} (glycosylated hemoglobin)
HDL-C	High Density Lipoprotein-Cholesterol
HPLC	High Performance Liquid Chromatography

IFG	Impaired fasting glucose
IGT	Impaired glucose tolerance
LDL-C	Low Density Lipoprotein-Cholesterol
LPO	Lipid Peroxidation
MAP	Mitogen Activated Protein
NADH	Nicotinamide Adenine Dinucleotide
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NO	Nitric Oxide
NPDR	Non Proliferative Diabetic Retinopathy
OGTT	Oral Glucose Tolerance Test
OS	Oxidative Stress
PDR	Proliferative Diabetic Retinopathy
PKC	Protein Kinase C
PUFA	Poly Unsaturated Fatty Acid
RAGE	Receptor for Advanced Glycosylation End product
RNS	Reactive Nitrogen Species
ROS	Reactive Oxygen Species
SD	Standard Deviation
TC	Total Cholesterol
TG	Triglycerides
TGF-P	Transforming Growth Factor-P
UKPDS	United Kingdom Prospective Diabetes Study
VLDL	Very Low Density Lipoprotein

ABSTRACT

BACKGROUND

Diabetes mellitus is known to induce oxidative stress along with deranging various metabolisms; one of the late complications of diabetes mellitus is diabetic retinopathy which is a leading cause of acquired blindness. Poor glycemic control, dyslipidemia and oxidative stress has been attributed to the development of complications like diabetic retinopathy.

OBJECTIVE

To study oxidative stress parameters (GSH & Vitamin C), HbA_{1c} and lipid profile in diabetic retinopathy patients and comparing the parameters and correlating the same with the controls.

MATERIAL AND METHOD

The study included 25 diabetic patients with retinopathy, 25 diabetic patients without retinopathy and 25 healthy controls, between 30 – 70 years of age of either sex, attending at R. L. Jalappa Hospital and Research Center, Kolar. Fasting blood glucose, HbA_{1c} and Lipid profile were measured by using standard methods adopted in the clinical laboratory. Glutathione in erythrocytes was assayed by colorimetric method using DTNB as a chromogen. Vitamin C was measured by colorimetric method using DNPH method.

The results obtained were analysed statistically by independent student‘t’ test & ANOVA.

RESULTS AND DISCUSSION

In our study, there was a significant increase in the FBS levels and HbA_{1c} levels in diabetic patients with retinopathy and in diabetic patients without retinopathy when compared to the control group. There was a statistically significant decrease in the mean Glutathione in erythrocytes and mean Vitamin C levels in the diabetic patients with retinopathy and in diabetic patients without retinopathy compared to the control group.

We found that there was statistically negative significant correlation between GSH with Total cholesterol & LDL cholesterol levels in diabetic mellitus group and there was statistically significant correlation between Vitamin C with FBS levels in control groups.

Key words: Diabetic retinopathy, HbA_{1c}, Lipid profile, Reduced Glutathione (GSH), Vitamin C.

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INTRODUCTION

Diabetes Mellitus is a complex metabolic disease, primarily characterized by hyperglycemia, caused by variable interactions between hereditary factors like HLA-DR_3, HLA-DR_4 and environmental factors like chemicals, cow's milk, virus, diet and exercise (i.e., by insulin resistance and or relative insulin deficiency).[1]

In persons with type II diabetes mellitus, complications are the major cause of morbidity and mortality which tend to worsen over time and carries a significant premature mortality risk. It's main features are abnormal insulin secretion & high levels of blood glucose, which are the major initiator of microvascular complications, including retinopathy, nephropathy, neuropathy and arteriosclerosis.[2]

These complications are predominantly seen in patients in the age group of 40 to 70 years. Poor glycemic control plays an important role in the development and progression of retinopathy and nephropathy with associated increase in morbidity and mortality.[3]

Several studies in this field have suggested a strong association between level of hyperglycemia and the progression of microvascular complications in diabetic patients.[4]

Chronic hyperglycemia and its associated non-enzymatic glycation play an important role in the development of microangiopathy. Intensive glycemic control as measured by serum HbA_{1c} levels have been demonstrated in randomised trials to reduce diabetic complications especially microvascular disease.[5]

Glycosylated hemoglobin levels reflect long term glycemic control and have proven to be a more accurate and stable measure than fasting blood glucose level.[6]

Diabetic retinopathy is a major cause of blindness in population of working age. It is one of the leading causes of acquired blindness in adults where the chances of loosing the sight are about 25 times higher than normal population. Decrease in visual acuity in diabetic retinopathy is either associated with maculopathy or proliferative complications of it. There are a series of risk factors related to the development and progression of diabetic retinopathy such as duration of diabetes mellitus, poor glycemic control, dyslipidemia and oxidative stress.[2]

Retinopathy is not only related to hyperglycemia and duration of diabetes mellitus but also related to the elevated serum lipids. There are some indications which show that elevated serum lipids promote retinopathy, especially by hard exudation. Studies have shown a positive correlation between serum cholesterol, low density lipoproteins and retinal hard exudates.[7]

Free radicals are chemical species possessing an unpaired electron that can be considered as fragments of molecules and which are generally very reactive. They are produced continuously in cells either as accidental by-products of metabolism or deliberately during phagocytosis. Cells have developed a comprehensive array of antioxidant defenses to prevent free radical formation or limit their damaging effects. Reactive free radicals formed within cells can oxidize biomolecules and lead to cell death and tissue injury.[8] Any tissue damage resulting from an imbalance between an excessive generation of oxidant compounds and insufficient antioxidant defense mechanism[9] is known as oxidative stress.

Recent studies have reviewed with evidence suggesting that, diabetes mellitus and its complications along with free radicals as a common pathway. The majority of reviews focus on the role of oxidative stress in development of diabetic complications.[10]

Several studies have been done to study the influence of these individual risk factors on the progression of retinopathy. However, very few studies have been done to study the correlation between all these risk factors in diabetic patients with retinopathy.

AIM OF THE STUDY:

An increase in pro-oxidant status over antioxidants causes oxidative stress affecting many bio molecules, mainly lipids. It is known that in the case of diabetes mellitus there is an increase in the levels of oxidative stress parameters. However, of the studies on oxidative stress parameters done in diabetes, only a few studies are done on diabetic complications like diabetic retinopathy.

This necessitates the present study for the estimation of oxidative stress parameters namely erythrocyte glutathione and vitamin C. The study also includes glycosylated hemoglobin, lipid profile (total cholesterol, triglycerides, LDL and HDL) and blood glucose levels. These parameters are done in subjects of diabetic retinopathy, diabetes without retinopathy and comparing them with age and sex matched healthy controls

OBJECTIVES OF THE STUDY

1. To study oxidative stress parameters namely erythrocyte glutathione and vitamin C levels, fasting blood glucose, glycosylated hemoglobin and lipid profile in serum of patients with diabetic retinopathy, diabetic without retinopathy and comparing the same with the controls.

2. To find out if there is any correlation between erythrocyte glutathione & vitamin C with fasting blood glucose, glycosylated hemoglobin and lipid profile in patients with diabetic retinopathy, diabetes without retinopathy and controls.

MATERIALS AND METHODS:

Source of data:

Study group: Total of 75 individuals divided into three groups.

Group 1: 25 Cases of type II diabetes mellitus with retinopathy.

Group 2: 25 cases of type II diabetes mellitus without retinopathy.

Group 3: 25 Normal individuals.

Inclusion criteria.

Clinically proven cases of type II diabetes mellitus with retinopathy based on fundoscopic changes between 30 – 70 years of age of either sex attending at Ophthalmology OPD, R.L. Jalappa Hospital and Research Center, Kolar were included in Group I. Diabetic subjects without retinopathy changes based on fundoscopic examination were included in Group II.

Exclusion criteria

Group-1

1. Non diabetic cases presenting with retinopathy.

2. Any subject with recent history of fever, infection and chronic illness like cancer, chronic obstructive lung disorders, cardiac diseases, stroke, gestational diabetic mellitus and complications related to diabetes like ulcers, neuropathy, nephropathy which are

known to affect oxidative stress parameters was excluded from the study. Any subject receiving antioxidant therapies were also excluded from the study.

Group- 2

Age and sex matched individuals with type II diabetes mellitus without diabetic retinopathy or other complications & antioxidant drugs**.**

Method of collection of blood:

1. After obtaining informed consent, 10ml of venous blood from the study and the control group were drawn under complete aseptic precautions.
2. One sample was collected in the fluoride tube after 8hrs of fasting and used for estimation of fasting blood glucose.
3. Another sample was collected in EDTA tube for glycated hemoglobin
4. Reduced Glutathione estimation was done in RBC which was obtained after centrifugation
5. Serum from the remaining sample was used for estimation of vitamin C and lipid profile.

Preparation of blood for analysis:

Blood sample from the anticoagulant [EDTA] containing vacutainer was centrifuged at 3000 rpm for 10 minutes; supernatant plasma was used for ascorbic acid estimation. The buffy coat was discarded. The packed cells were suspended in equal volume of cold phosphate buffer saline and re-centrifuged. The supernatant was discarded. The washing

of packed cells was repeated twice, the packed cells were used for analysis of glutathione.

Estimating Parameters:

The parameters were estimated using the following methods:

1. Blood glucose by glucose oxidase enzymatic method.
2. Glycated hemoglobin by cation exchange resin method.
3. Total cholesterol by cholesterol oxidase - peroxidase method
4. Triglycerides by enzymatic method using glycerol-3-phosphate as substrate
5. HDL cholesterol by precipitation (with phosphotungstic acid) method.
6. LDL cholesterol using Friedewald's formula

$$LDL = TC - (HDL + TG/5)$$

7. Erythrocyte reduced glutathione will be assayed by colorimetric method using 5,5′-Di Thiobis 2-Nitrobenzoic Acid (DTNB) as a chromogen
8. Vitamin C measured by colorimetric by non-enzymatic method, using 2, 4 – Dinitrophenyl hydrazine (DNPH)

ESTIMATION OF BLOOD GLUCOSE

Method: Enzymatic Colorimetric End point test (GOD –POD).[12]

Accucare kit. (Lab Care Diagnostics Pvt Ltd, India)

Principle:

Glucose is determined after enzymatic oxidation in presence of glucose oxidase. The hydrogen peroxide formed reacts with phenol and 4-aminophenazone to form a red violet quinoeimine dye as indicator.

Reagents:

Reagent 1 Enzyme reagent

Glucose Standard: 100mg/dl

Sample: Serum or plasma

Automated Parameters:

Wavelength: 505nm

Reaction type: End point

Cuvette: 1cm light path

Temperature: 37^0C

Reaction type: Increasing

Measurement: Against reagent blank

Sample/reagent ratio: 1:100

Incubation: 5mins

Max blank Abs: 0.30

Low normal: 60mg/dl

High normal: 110mg/dl

Linearity: Up to 400mg/dl

Manual Procedure: Pipette into test tubes

	Blank	**Standard**	**Sample**
Sample	-	-	10µl
Std	-	10µl	-
Reagent	1000µl	1000µl	1000µl

Mix and incubate for 5mins at 37^{o}C or 15mins at room temperature. Measure the absorbance of the sample (AT) and standard (AS) against reagent blank at 505nm. The colour is stable for 30min at room temperature.

Calculation:

Total glucose (mg/dl) = AT/AS X conc. STD

Reference Values: Fasting blood sugar: 60-110 mg/dl

ESTIMATION OF GLYCOSYLATED HEMOGLOBIN (HBA_{1C}):

Method: Cation-exchange resin method.[11]

(Recombigen Laboratories Pvt Ltd, India)

Principle:

Whole blood is treated with lysing reagent to prepare a hemolysate. This is then mixed with a weakly binding Cation-exchange resin. The non-Glycosylated hemoglobin binds to the resin leaving Glycosylated hemoglobin (GHb) free in the supernatant. The GHb percentage is determined by measuring the absorbance at 415nm (405-420nm acceptable) of the GHb fraction and of the total Hb. The ratio of the two absorbances gives the glycosylated hemoglobin percent.

Reagents:

1. Resin reagent: 8 mg/ml cation exchange resin buffered at pH 6.9.
2. Lysing reagent: 10 mm potassium cyanide surfactant.
3. Glycosylated hemoglobin standard: 10% glycosylated hemoglobin.
4. Serum separators.

Procedure:

1. Hemolysate preparation:

a. .500 µl lysing reagent is dispensed to tubes labeled: standard, control and sample 1 etc.

b. Place 100 µl of well-mixed blood sample standard or control into the appropriately labeled tube.mix well.

c. Tubes are allowed to stand for 5 minutes.

2. Glycosylated hemoglobin preparation:

a. 3.0 ml of Glycosylated hemoglobin cation-exchange resin is dispensed into 13×100mm glass tubes labeled: standard, control, sample 1 etc. Note: Before use, mix the resin by inverting at least 10 times. Swirl the bottle after addition to each tubes.

b. 100 µl of hemolysate is added (from step A3).

c. Position the filter separators in the tubes so that the rubber sleeve is approximately 1 cm above the liquid level.

d. The tubes are placed on the rocker or rotator and continuously mixed for 5 minutes.

e. The tubes are removed from the rocker or rotator.

f. Push the filter separators into the tubes until the resin is firmly packed.

g. The supernatant may be poured into another tube or directly into a cuvette for absorbance measurement.

h .Absorbance is set to zero at 415 nm (405-420) with deionised water as the blank.

i. Read and record the absorbance values for standard, control, sample 1 etc.

3. Total hemoglobin fraction:

a. 5 ml of deionised water is dispensed into plastic or glass tubes labeled: standard, control, sample 1 etc.

b. 20 µl of the hemolysate (from step A3) is placed into the appropriately labeled tube. Mix well.

c. Adjust the instrument to zero absorbance at 415 nm (405-420) with deionised water as the blank.

d. Read and record the absorbance values for standard, control, sample 1 etc.

These recordings are for total hemoglobin.

Calculation:

Results for the unknowns and controls are calculated as follows: For each sample, calculate the ratio (R) of the Glycosylated hemoglobin absorbance to the total hemoglobin absorbance. Use the following equation to determine unknown concentration:

Unknown (%) = R (Unk) / R (STD) ×STD Conc (%).

$$\text{Absorbance of Std} = \frac{\text{Absorbance of Std GHb}}{\text{Absorbance of Std THb}} = A1 = 1.7(*)$$

$$\text{Absorbance of Sample} = \frac{\text{Absorbance of Sample GHb}}{\text{Absorbance of Sample THb}} = A2$$

Unknown (%) GHb in Sample = A2 /A1 ×10

Expected Values:

Non Diabetic: 4.5% - 8.0%

Good control: 8.0% - 9.0%

Fair control: 9.0% - 10.0%

Poor control: 10.0% & above.

ESTIMATION OF TOTAL CHOLESTROL

Method: Enzymatic CHOD-PAP method.[13]

(Recombigen Laboratories Pvt Ltd, India)

Principle:

Cholesterol esterase hydrolyzes cholesterol esters to free cholesterol and fatty acids. Cholesterol is oxidized by cholesterol oxidase forming hydrogen peroxide and cholest-4ene-3one. In presence of peroxidase, hydrogen peroxide formed brings about oxidative coupling of phenol and antipyrine to form red colored quinoneimine dye.

$$\text{Cholesterol ester} + H_2O \xrightarrow{\text{Cholesterol esterase}} \text{Cholesterol} + \text{fatty acid}$$

$$\text{Cholesterol ester} + O_2 \xrightarrow{\text{Cholesterol oxidase}} \text{Cholest-4ene-3one} + H_2O_2$$

$$2H_2O_2 + \text{4- aminoantipyrine} + \text{Phenol} \xrightarrow{\text{Peroxidase}} \text{Quinoneimine dye} + 4H_2O_2$$

Reagent Composition:

Pipes buffer, pH 6.70:	50mmol/L
Phenol:	24mmol/L
Sodium cholate:	0.5mmol/L
4-Aminoantipyrine:	0.5mmol/L
Cholesterol esterase:	180U/L
Cholesterol oxidase:	200U/L
Peroxidase:	1000U/L
Standard cholesterol:	200mg/dl

Procedure:

Wavelength: 500nm

Cuvette: 1cm light path

Read against reagent Blank

Pipette into test tubes

	Blank	**Standard**	**Sample**
Reagent	1ml	1ml	1ml
Distilled water	10µl	–	–
Standard	–	10µl	–
Sample	–	–	10µl

Mix and read the OD after 5mins incubation. The final color is stable for at least one hour

Calculations:

$$\text{Concentration of total cholesterol} = \frac{\text{Absorbance (sample)}}{\text{Absorbance (standard)}} \text{ x } 200$$

= ……..mg/dl

Reference Value: 150-260mg/dl.

ESTIMATION OF SERUM TRIGLYCERIDES

Method: Enzymatic method (GPO-PAP method).[13]

(Recombigen Laboratories Pvt Ltd, India)

Principle:

Triglycerides are hydrolyzed by lipoprotein lipase to glycerol and fatty acids. Glycerol is first phosphorylated to glycerol-3-phosphate by glycerol kinase and then oxidized by glycerol phosphate oxidase forming hydrogen peroxide and dihydroxy acetone phosphate. This hydrogen peroxide in the presence of peroxidase causes oxidative coupling of 4- chlorophenol and 4- aminoantipyrine to form red colored quinoneimine dye which is measured at 505 nm. The decrease in absorbance is directly proportional to the concentration of triglycerides.

$$\text{Triglycerides} + 3H_2O \xrightarrow{\text{Lipoprotien lipase}} \text{Glycerol} + 3 \text{ free fatty acids}$$

$$\text{Glycerol} + \text{ATP} \xrightarrow[Mg^{2+}]{\text{Glycerol kinase}} \text{Glycerol 3 Phosphate} + \text{ADP}$$

$$\text{Glycerol 3 Phosphate} + O_2 \xrightarrow{\text{GPO}} \text{Dihydroxy acetone phosphate} + H_2O_2$$

$$H_2O_2 + \text{4 aminoantipyrine} + \text{4 chlorphenol} \xrightarrow{\text{Peroxidase}} \text{Quinoneimine} + HCl + H_2O$$

Reagent Composition:

Pipes buffer, pH 7.0:	50 mmol/L
P-Chlorophenol:	5.3mmol/l

Potassium ferrocynate: 10µmol/l

Magnesium salt: 17mmol/l

4-Aminoantipyrine: 0.9mmol/l

ATP: 3.15mmol/l

Lipoprotein lipase: ≥1800 U/L

Glycerol kinase: ≥450 U/L

Glycerol-3-phosphate oxidase ≥3500 U/l

Peroxidase: ≥450 U/l

Standard: 200 mg/dl

Procedure:

Wavelength: 500nm

Temperature: 37^{o}C

Cuvette: 1cm light path

Read against reagent blank

Pipette into test tubes

	Blank	**Standard**	**Sample**
Reagent	1ml	1ml	1ml
Distilled water	10µL	-	-
Standard	-	10µL	-
Sample	-	--	10µL

Mix and read the OD after 10mins of incubation.

Calculations:

$$\text{Triglycerides (mg/dl)} = \frac{\text{Absorbance of Test}}{\text{Absorbance of Standard}} \text{ X Concentration of Standard}$$

.....mg/dl

Reference Values:

Males: 60-165mg/dl

Female: 40-140mg/dl

ESTIMATION OF SERUM HDL CHOLESTEROL

Method: Enzymatic CHOD-PAP method.[13]

(Colorimetric end point test, by using Accucare kit, Labcare PVT Ltd, India)

Principle:

Chylomicrons, VLDL and LDL are precipitated by adding phosphotungstic acid and magnesium ions to sample. Centrifugation leaves only HDL in the supernatant. The cholesterol content in it is determined enzymatically.

Reagent:

Precipitating reagent:

Phosphotungstic acid	2.4 mmol/L
Magnesium chloride	40 mmol/L

HDL cholesterol Standard – 50 mg/dl

Procedure:

1) Precipitation Step:

Pipette into centrifuge tubes

Sample	**Reagent**
500µl	500µl

Mix and allow it to stand for 5mins. Centrifuge for 10mins at 3000rpm and determine the cholesterol content using supernatant by the CHOD-PAP method.

2) Assay Procedure:

Pipette in to tubes marked	Blank	Standard	Test
Cholesterol working reagent	1000 µl	1000 µl	1000 µl
Distilled water	50 µl	--	--
HDL standard	--	50 µl	--
Supernatant	--	--	50 µl

Mixed well, incubated for 05 minutes at 37^0C (10 minutes. at 20 -25^0C). Read the absorbance of the standard and each test at 520 nm against reagent blank. The colour is stable for 90 minutes at 20 -25^0C.

Automated Parameters:

Wavelength: 520nm

Reaction type: End point

Cuvette: 1cm light path

Reaction temperature: 37^oC

Measurement: against blank

Sample/reagent ratio: 1:20

Incubation: 5mins

Maximum reagent blank Abs: 0.30

Low normal at 37^oC: 41mg/dl

High normal at 37^oC: 75mg/dl

Calculation:

$$\text{HDL Cholesterol (mg/dl)} = \frac{\text{Absorbance of Test}}{\text{Absorbance of Standard}} \text{ X Concentration of Standard (mg/dl) X Dilution factor}$$

$$= \frac{\text{Absorbance of Test}}{\text{Absorbance of Standard}} \text{ x50 x 2}$$

$$= \frac{\text{Absorbance of Test}}{\text{Absorbance of Standard}} \text{ x100}$$

= ---------- mg/dl

Reference Values:

Men: 41.0 - 58.7mg/dl

Women: 48.8 - 75.0mg/dl.

CALCULATION OF SERUM LDL-CHOLESTEROL AND

VLDL-CHOLESTEROL BY FRIEDEWALD FORMULA[13]

Values required: 1) Total cholesterol.

2) HDL.

3) Triglycerides.

$$\text{LDL Cholesterol} = \text{Total Cholesterol} - \frac{\text{Triglycerides}}{5} - \text{HDL cholesterol}$$

Limitations:

(i) No chylomicrons in sample.

(ii) Triglyceride concentration not more than 400 mg/dl.

$$\text{VLDL Cholesterol} = \frac{\text{Triglycerides}}{5}$$

ESIMATION OF OXIDATIVE STRESS PARAMETERS

ESTIMATION OF GLUTATHIONE (GSH) IN ERYTHROCYTES

Method: By colorimetric method using 5,5'- Di Thiobis 2-Nitrobenzoic Acid (DTNB) as a chromogen.[14]

Principle:

The major non-protein sulfhydryl groups of RBC's are in the form of reduced GSH.5, 5′ dithiobis 2-nitrobenzoic acid (DTNB) is a disulfide chromogen that is readily reduced by sulfhydryl compounds to an intensely yellow compound. The absorbance of the reduced chromogen is measured at 412 nm and is directly proportional to GSH concentration.

Reagents:

1. Precipitating Reagent: (Stable for 3 weeks at 4^0C)

   1.67gm of glacial metaphosphoric acid, 0.2 gm of Na/K salt of EDTA and 30 gm of NaCl in 100 ml of Distilled water.

2. 0.3M Phosphate Solution:(Stable indefinitely at 4^0C)

   4.25 gm of Na_2HPO_4 in 100ml of distilled water.

3. DTNB solution: (stable for 13weeks at 4^0C) Di Thio bis Nitro Benzoic acid.

   40 mg DTNB + 100ml of 1 % sodium citrate solution, Stored in a dark bottle.

4. Sodium citrate solution: 1g of Sodium citrate in 100ml of distilled water.

5. Glutathione standard: (freshly prepared)

   5mg GSH/10 ml of water.

Procedure:

1. 0.2ml of packed cells was taken in centrifuge tubes.
2. 1.82ml of distilled water was added to it.
3. Out of the above 2.020ml solution, 0.02ml (20µl) was taken add 4ml of Drabkins hemoglobin solution and absorbance was measured at 540 nm after 5min.
4. To the rest 2ml, 3ml of precipitating reagent was added, mixed and allowed to stand for 10minutes.The solution was centrifuged after 10 minutes and filtered.
5. 1ml of filtrate was taken and 4ml 0.3M Phosphate solution was added. 1ml of water instead of supernatant was used as blank and 4ml 0.3M Phosphate solution was added to it.
6. The tubes were vortexed properly and 0.5ml DTNB was added.
7. Absorbance was measured at 412nm within 5 minutes.

CONTENTS	**B**	**S_1**	**S_2**	**S_3**	**S_4**	**S_5**	**S_6**	**S_7**	**T**
Vol of Std (ml)	-	0.1	0.15	0.20	0.25	0.30	0.35	0.40	-
Conc of Std(ug)	-	50	75	100	125	150	175	200	-
Vol of D/W (ml)	2	1.9	1.85	1.80	1.75	1.70	1.65	1.60	-
Vol of ppt Reagent(ml)	3	3	3	3	3	3	3	3	3
Vol of filtrate(ml)	1	1	1	1	1	1	1	1	1
Vol of 0.3M Phosphate solution(ml)	4	4	4	4	4	4	4	4	4
Vol of DTNB (ml)	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Absorbance at 412 nm	0.0	0.02	0.03	0.04	0.05	0.07	0.07	0.08	

Carry out with each tube (one at a time), add DTNB within 1 minute.

Calculations:

Hemoglobin:

$$\text{Hb gm/dl} = \frac{\text{O.D. x Extinction co efficient of product x Dilution factor}}{10}$$

$$= \frac{\text{O.D. X 1.47 x 201}}{10}$$

Hb gm/dl = O.D. X 29.547

Hb gm/dl = X

Dilution factor:

0.02ml + 4ml Drabkins solution =Total 4.02ml

So dilution factor = 201 (1/0.02 X 4.02 = 201)

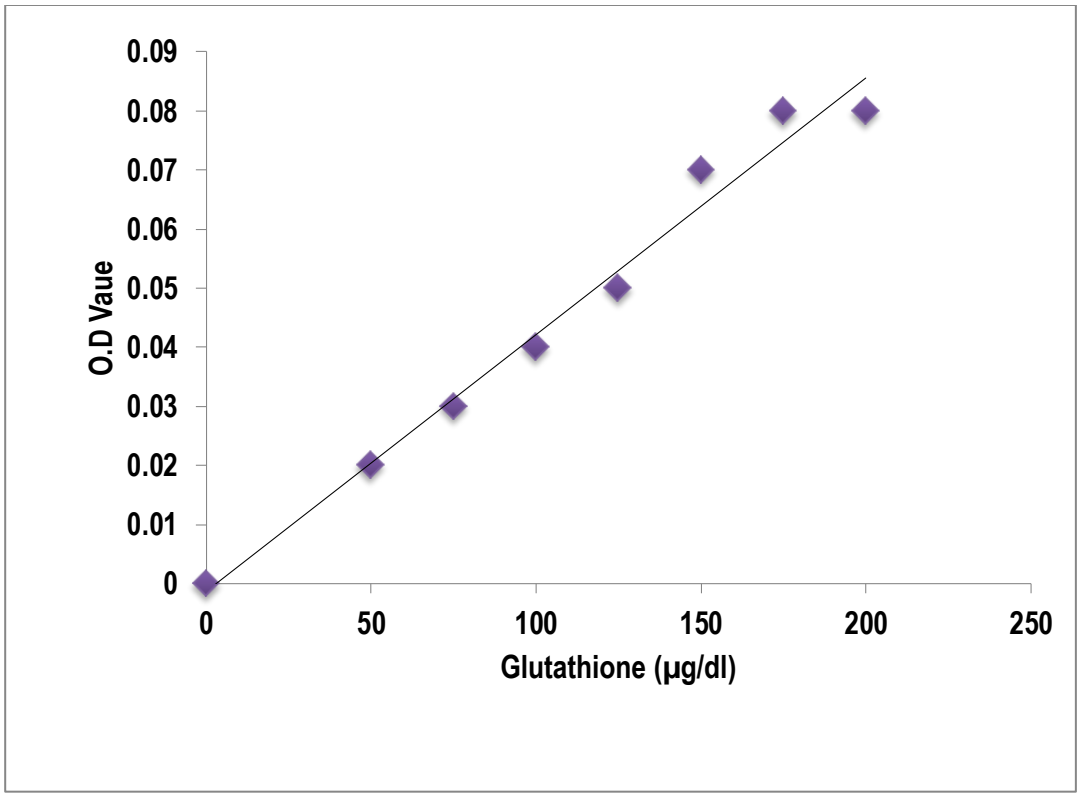
GSH Concentration is interpreted in micro gram from the graph

$$\text{GSH (mg/ dl of packed cells)} = \frac{\mu \text{ gram (concentration) X 100(ml of packedcells)}}{0.2 \text{ X } 1000}$$

$$= \frac{\mu \text{ gram}}{2}$$

GSH mg/ dl of packed cells =Y

GSH mg/gm of HB = Y/X



Graph No 1: Standard graph for GSH

ESTIMATION OF ASCORBIC ACID (VITAMIN C) IN PLASMA

Method: By colorimetric, nonenzymatic method, using 2, 4 – Dinitrophenyl hydrazine (DNPH).[15]

Principle:

Ascorbic Acid in plasma is oxidized by bromine to form dehydro-ascorbic acid, which reacts with 2,4 - dinitrophenyl hydrazine to form red bis-hydrazone which is measured at 520 nm. (Sample should be analysed immediately or not later than 3hrs if the specimen is refrigerated).

Reagents:

1. Metaphosphoric Acid Solution – 4% (4 gms of Metaphosphoric Acid in 100 ml distilled water, Prepare immediatel before use)
2. 85% Sulphuric acid, Refrigerate
3. 2, 4 Dinitrophenyl Hydrazine (DNPH) – 2 gms/dl, 2 gms of DNPH in 100 ml of 9N sulphuric acid (24.48ml to 100ml).Let stand in the refrigerator overnight & then filter.
4. Liquid bromine
5. Thiourea Solution – 10gms/dl, 10 gms of thiourea in 50% alcohol. Reagent is stable for one month at 4^{o} C.
6. Ascorbic acid stock standard: All ascorbic standards should be prepared daily.100 mg of Ascorbic Acid in 100 ml of 4% Metaphosphoric acid.
7. Ascorbic acid working standard: By diluting 2.5ml of stock solution with 17.5ml of 4%.

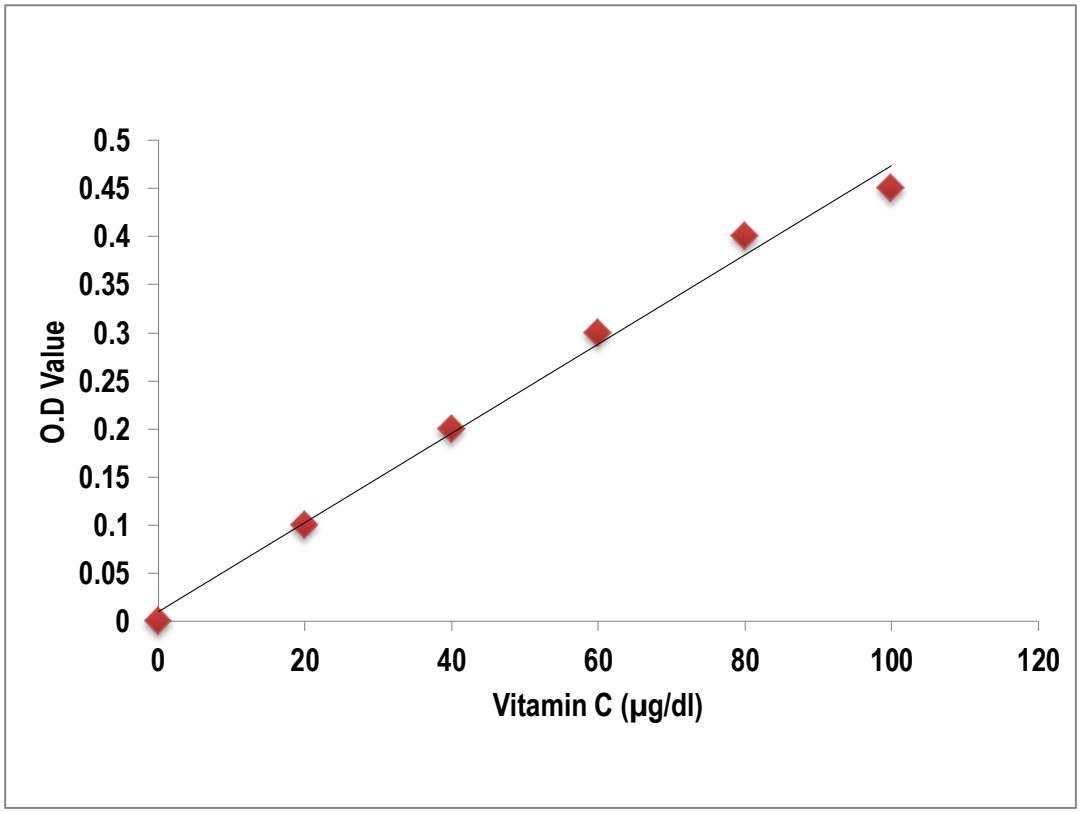
8. Metaphosphoric acid. Two drops of bromine is added. Kept in ice chest for 2 hrs. Excess bromine is removed by aeration. After aeration, the contents are transferred to 100ml standard flask and make it upto 100ml with 4% Metaphosphoric acid.

Procedure:

CONTENTS	**B**	**S_1**	**S_2**	**S_3**	**S_4**	**S_5**	**T**
Vol of Std(ml)	-	0.8	1.6	2.4	3.2	4.0	-
Conc of Std(ug)	-	20	40	60	80	100	-
Vol of Test(ml)	-	-	-	-	-	-	0.5
Vol of 4% Metaphosphoric acid(ml)	4	3.2	2.4	1.6	0.8	-	3.5
Vol of Thiourea(drop)	1	1	1	1	1	1	1
Vol of 2,4-DNPH (ml)	1	1	1	1	1	1	1
Incubate at37^0 C for 3 hrs, later cool in ice chest.							
Vol of 85%H_2SO_4 (ml)	5	5	5	5	5	5	5
Absorbance at 530 nm	0.00	0.10	0.20	0.30	0.40	0.45	

Calculation:

Concentration of Sample Ascorbic Acid is obtained from the Standard Curve in micro gram and is converted to milligrams, gives the concentration of Ascorbic Acid/dl of plasma.



Graph No 2: Standard graph for Ascorbic Acid.

HISTORY OF DIABETES

Diabetes is one of the oldest diseases known to mankind. Its various features have been described by physicians of ancient civilizations in countries like Greece, India, China and Egypt. The first mention of a diabetes mellitus like condition is found in an Egyptian Papyrus (Papyrus EBERS), dating back to about 1500BC, in which polyuria has been described. Chinese physician Nezling in 400BC and Greek physician Celsus (30BC to 50AD) have also described important symptoms of diabetes. Charaka in 2nd century AD mentioned the sweetness of urine in addition to polyuria. Sushrutha, around 5th century AD, gave a comprehensive description of the disease, which he called "Madhumeha" (rain of honey), because of sweet taste of urine in these patients, attracting ants and other insects. In 2nd century AD, Aretaeus of Cappedocia in Greece is credited to have coined the term "Diabetes" conveying excessive thirst and polyuria.[16]

In the medieval period, Chen Chhuan in 7th century AD and Arabian physician Avicenna described certain complications of diabetes like sexual dysfunction and gangrene.[16]

With the advent of modern era, Thomas Willis in 16th century called this condition as the "pissing evil" due to the troublesome polyuria and complications caused by it. Mathew Dobson in 1776 explained sweetness of urine due to presence of sugar. He evaporated the samples of urine and the residues which were left looked like brown sugar. In 1850 Claude Bernard proposed 'hyperglycemia' as the cardinal symptom of diabetes. In 1879, Paul Langerhans submitted his thesis to Berlin University in which he

described the presence of different kind of tissue scattered among the acini throughout the pancreatic gland – the islets of langerhans.

The role of pancreas in digestion was established by Von Mering and Minkowski in 1889. They surgically removed the pancreas from two dogs. By the next day both dogs manifested with polyuria. Diabetes was diagnosed on testing urine for sugar. Minkowski also demonstrated that grafting a piece of freshly removed pancreas under the skin of dogs prevented appearance of glycosuria as long as the graft was intact. Till 1900, biochemical tests to detect diabetes were not available. Benedict in 1911 published his method for detection and estimation of reducing sugars, mainly glucose, in urine. Rothera devised a technique for detection of ketone bodies in urine during first decade of 20^{th} century.[16]

In 1902 Bayliss and Starling proposed the term "hormone" to non exocrine regulatory secretions of the duodenum. The hypothetical anti-diabetic hormone from pancreas was named "insulin" by Sharpey-Schafer in 1916, and the hormone was isolated by Banting and Best in 1922. A monograph on the visual problems in diabetes was published in 1935 by Waite and Beetham. Beetham later studied the natural history of retinopathy. In 1936, Kimmelstiel and Wilson's article on a kidney lesion that seemed pathognomonic for diabetes rounded out the early description of diabetic complications.

DIABETES MELLITUS:

Diabetes Mellitus is a complex metabolic disease caused by a variable interaction between hereditary and environmental factors.[2] It is one of the most common metabolic,

lifelong progressive disease in which either the body's inability, to produce insulin or use insulin, to its full potential and is characterized by high circulating glucose.[17]

Type I diabetes mellitus results from cellular-mediated autoimmune destruction of β cells of Islets of Langerhans of pancreas and results in loss of insulin production, accounting for 5% to 10% of cases of diabetes. Type 2 diabetes mellitus is characterized by Insulin resistance and abnormal insulin secretion. Type 2 diabetes mellitus is the most common form of diabetes mellitus accounting for 90% of cases.[18] Traditionally Type 2 diabetes mellitus is considered as a disease of adult Type 2 diabetes is increasingly diagnosed in children in parallel to rising obesity rates.[19]

Diabetes Mellitus is one of the main threats to human health, as one of the most challenging health problems of the 21st century. It affects more than 230 million people worldwide, and this would increase to 350 million by the year 2025. Increasing urbanization and industrialization are the chief reasons for the rapid increase in the prevalence of type 2 diabetes mellitus.[17]

In recent years, India has witnessed a rapidly exploding epidemic of diabetes mellitus and it leads today, with the largest number of diabetics in any given country of the world. It has been estimated that in 1995, 19.4 million individuals were affected by diabetes mellitus and these numbers were expected to rise to 57.2 million by the year 2024.[20]

Diabetes Mellitus is a common and potentially disabling chronic disease and sustained hyperglycemia attacks both microvessels and macrovessels throughout the body. Persons with diabetes mellitus are at an increased risk of number of complications

including retinopathy, renal disease, heart disease and neuropathy. These complications can progress to end stage outcomes such as blindness, visual impairments, end stage renal disease and amputation.[17]

These complications are believed to be major contributors to mortality and morbidity in patients with diabetes. Diabetes mellitus associated complications can be delayed or prevented with continuous glycemic control, accomplished by, better education, improved nutrition, increased physical activity, weight reduction; early diagnosis and prompt treatment are all means of curbing the impact of diabetes mellitus.[18]

CLASSIFICATION OF DIABETES

Diabetes mellitus is classified as different types as follows.[12]

Type 1 diabetes mellitus • Immune mediated • Idiopathic
Type 2 diabetes mellitus
Other specific type of diabetes mellitus Genetic defects of islet β- cell function • Genetic defects in insulin action diseases of exocrine pancreas • Endocrinopathies • Drug-and chemical- induced diabetes • Infections • Uncommon forms of diabetes • Other genetic syndromes
Gestational diabetes mellitus

Table No 1: Classification of Diabetes.

PATHOGENESIS OF DIABETES MELLITUS (DM)

Type I-Diabetes Mellitus.

Develops as a result of synergistic effects of genetic, environmental & immunological factors, which ultimately destroy the pancreatic beta cell giving rise to complete insulin deficiency.[21]

In Type I diabetes mellitus the symptoms of hyperglycemia appears sudden and is often severe, by the time it appears, most of the beta cells in the pancreas would have been destroyed. The destruction is almost certainly autoimmune in nature. Pathogenesis in Type I begins with glucose susceptibility to the disease and some environmental events initiates the process in such susceptible individuals (e.g. viral infection may be one such triggering factor). Once the stimulus is given, autoimmune attack then follows, although the process is clinically silent, the islets become infiltrated by monocytes/macrophages and activated cytotoxic T cells. This infiltration is usually designated as "insulitis". Further as the autoimmune destruction of islets cells continues, insulin secretion is affected and diabetes eventually sets in.[21]

Type II-Diabetes Mellitus:

Type II diabetes mellitus develops due to insulin resistance, abnormal insulin secretion & increased hepatic glucose production. The fundamental molecular defects in insulin resistance & insulin secretion result from a combination of environmental & genetic factors.[21]

Type II, which is more common than Type I, frequently exhibit familial aggregation; its pathogenesis is less well understood. Nevertheless, both beta cell defects and insulin

resistance are present in overt disease and the major environmental factor is obesity. Patients with Type II diabetes mellitus have two pathophysiological defects, abnormal insulin secretion and resistance to insulin action in target tissue. Descriptively, the phase can be recognized in the usual clinical sequences. In the first phase, the plasma glucose remains normal despite demonstrable insulin resistance because insulin levels are elevated. In the second phase, insulin resistance tends to worsen so that postprandial hyperglycemia develops despite elevated insulin concentration. In the third phase, insulin resistance does not change, but declining insulin secretions causes fasting hyperglycemia and overt diabetes.[21]

METABOLIC DERANGEMENTS IN DIABETES MELLITUS

Carbohydrate Metabolism:

In cases of diabetes mellitus, carbohydrate metabolism is deranged due to insulin deficiency, which decreases the uptake of glucose by adipocytes & skeletal muscles. Net effect of this causes inhibition of glycolysis & stimulation of gluconeogenesis, leading to hyperglycemia.[12]

Protein Metabolism:

In diabetes mellitus, protein metabolism is deranged due to increased breakdown of proteins & aminoacids which are substrates for gluconeogenesis, leading to muscle wasting. The absence of anabolic effect of insulin enhances the muscle wasting.[12]

Lipid Metabolism:

To meet the energy requirements in diabetes mellitus,there is breakdown of fatty acids, leading to increased levels of free fatty acid in plasma which further leading to fatty

liver. Excess of acetyl CoA, which is formed cannot be efficiently oxidized by TCA cycle, due to limited availability of oxaloacetate, because of stimulation of gluconeogenesis, which is mainly responsible for the depletion of oxaloacetate. The excess acetyl CoA is diverted to the formation of ketogenesis. Net effect resulting in hyperlipidemia.[12]

CLINICAL MANIFESTATIONS:

Glucosuria, polyuria, polydypsia, polyphagia, loss of weight, recurrent infections, microangiopathy (particularly affecting retina, kidneys) and macroangiopathy (e.g. ischemia heart disease, cerebrovascular disease).

DIAGNOSTIC CRITERIA

In 1979 and 1980, two groups, the National Diabetes Data Group in the United States and the World Health Organization (WHO), published reports addressing diabetes diagnostic criteria.[22,23] The two groups reached the same conclusions and the criteria were eventually adopted throughout the world. Before that time, criteria were variable and established by research groups and clinicians using their individual clinical experiences and limited data.

In 2003, the Expert Committee published a follow-up report in which it carefully considered new data since its 1997 report.[24]

This follow-up report retained the previous fasting and 2-hour diagnostic criteria and the Impaired Glucose Tolerance (IGT) criteria. However, the committee changed the Impaired Fasting Glucose (IFG) criteria to 100–125 mg/dl, lowering the previous lower threshold of 110 mg/dl. The evidence that supported this change came from four large

U.S. and international population-based observational studies finding that IFG defined as 100–125 mg/dl predicted future diabetes (by either fasting or 2-hour criteria) better than IFG defined as 110–125 mg/dl. This change in IFG criteria has been estimated to increase the proportion of the population affected by two- to fivefold, depending on demographic subgroup.[25]

Criteria	1979 and 1980	1997 and 1999	2003
Fasting plasma glucose			
Diabetes	≥140mg/dl	≥126mg/dl	≥126mg/dl
IFG	**	100-125mg/dl	100-125mg/dl
2-hour plasma glucose*			
Diabetes	≥200mg/dl	≥200mg/dl	≥200mg/dl
IFG	140-199mg/dl	140-199mg/dl	140-199mg/dl
2-hour 75gm OGTT ****Not considered***.			

Table No 2: Diagnostic Criteria for Diabetes & Impaired Glycemic States

PATHOGENESIS OF DIABETIC COMPLICATIONS

Hyperglycemia is considered a major factor in the development of diabetic complications and the adverse effects are recognizable through multiple pathways.

As hyperglycemia is the major cause for diabetes mellitus and its complications, various theories have been put forward. Some of the accepted theories are:

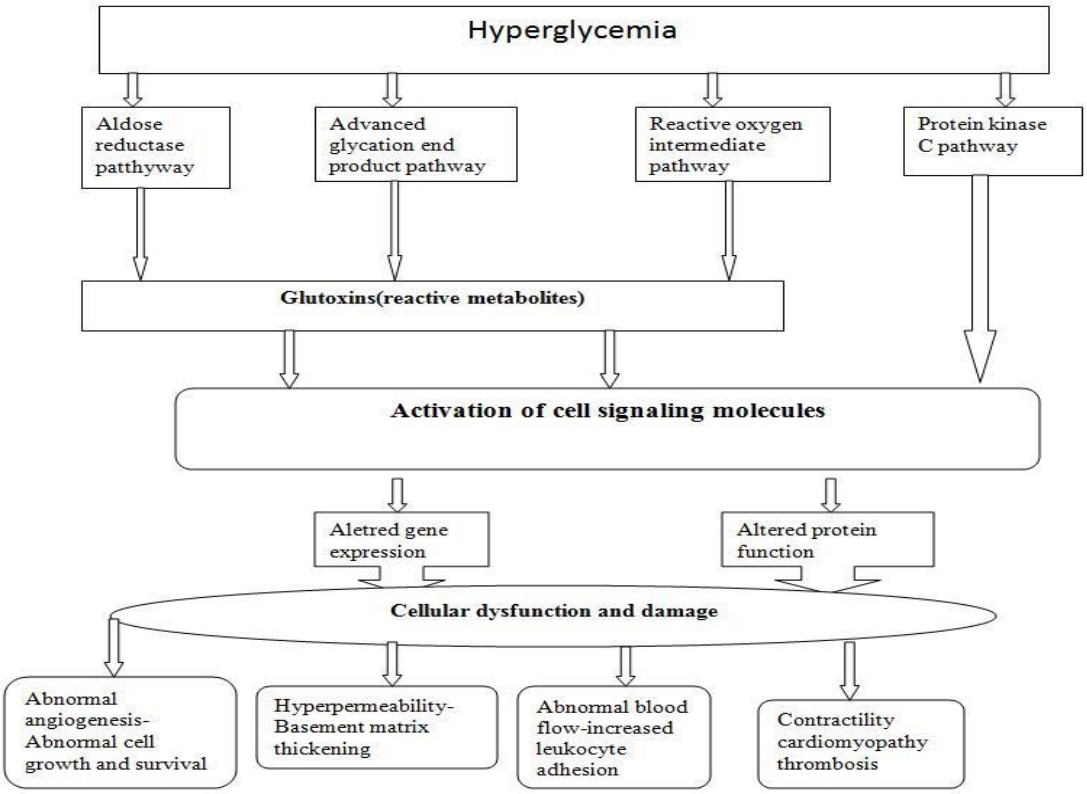


Figure No 1: Pathogenesis of diabetic complications

1. **Aldose reductase (polyol pathway) theory**:

Hyperglycemia causes an increased flux through the enzyme aldose reductase which gets activated and uses nicotinamide adenine dinucleotide phosphate hydrogenase (NADPH) to reduce glucose to sorbitol. This is then oxidized to fructose via sorbitol dehydrogenase. The decline in NADPH caused by increased aldose reductase flux decreases the generation of nitric oxide in endothelial cells[26] and cellular redox balance. Increased NADH/NAD^+ ratio that may alter enzyme activity also contribute to the complications, by increased sorbitol accumulation which is neurotoxic to nerves

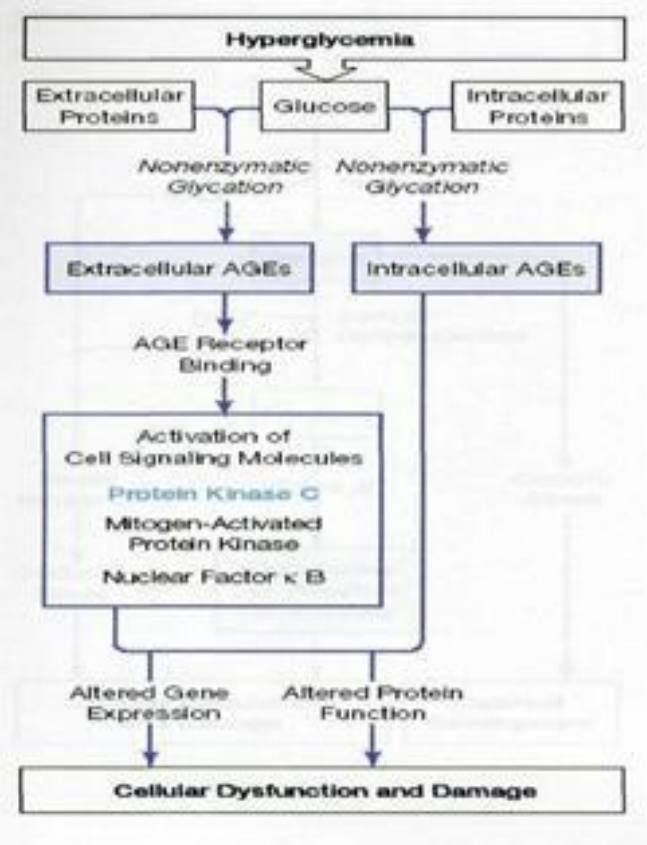


Figure No 2: Aldose reductase pathway

2. **Advanced Glycation End Product (AGEs) theory:**

During the normal course of ageing, proteins become irreversibly modified by sugar in a process known as Mailard reaction, leading to "tissue browning". Hyperglycemia in diabetes accelerates this process by covalent modification and crosslinking proteins.[28] The products of the nonenzymatic glycation of proteins are varied in chemical structure and as a group, have been termed AGEs. Formation of AGEs may damage cells by impairing function of a wide range of proteins including modifications of extracellular structural proteins such as collagen and intracellular proteins.[29] AGEs can also alter cellular function by binding to receptors called Receptor for AGEs (RAGE), a transmembrane receptor. This initiates a cascade of cellular signaling events, such as

activation of Mitogen Activated Protein (MAP) kinase which can lead to cellular dysfunction.[30]

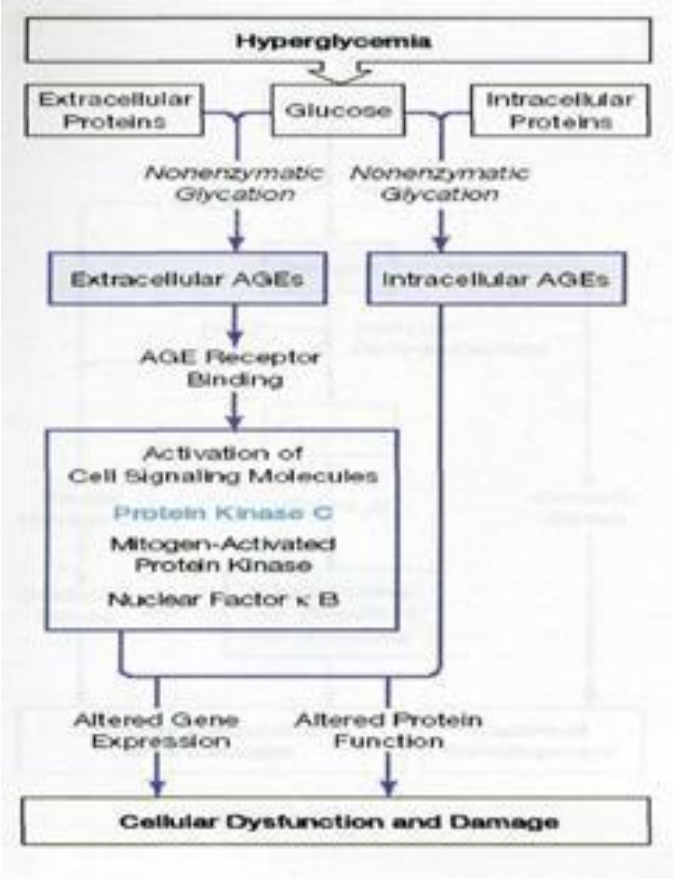


Figure No 3: Advanced glycation endproduct Pathway

3. Reactive oxygen intermediate theory:

The metabolism of glucose through glycolytic pathway and the tricarboxylic acid cycle produces reducing equivalents that are used to drive ATP synthesis via oxidative phosphorylation in the mitochondria. Byproducts of mitochondrial oxidative phosphorylation include free radical such as superoxide anion and their generation is increased by high glucose levels.[31] Glucose autooxidation also creates free radicals that can damage cellular proteins as well as mitochondrial DNA.[32] Increased oxidant stress

reduces nitric oxide levels,[33] damages cellular proteins and promotes leucocyte adhesion to the endothelium while inhibiting its barrier function.[34]

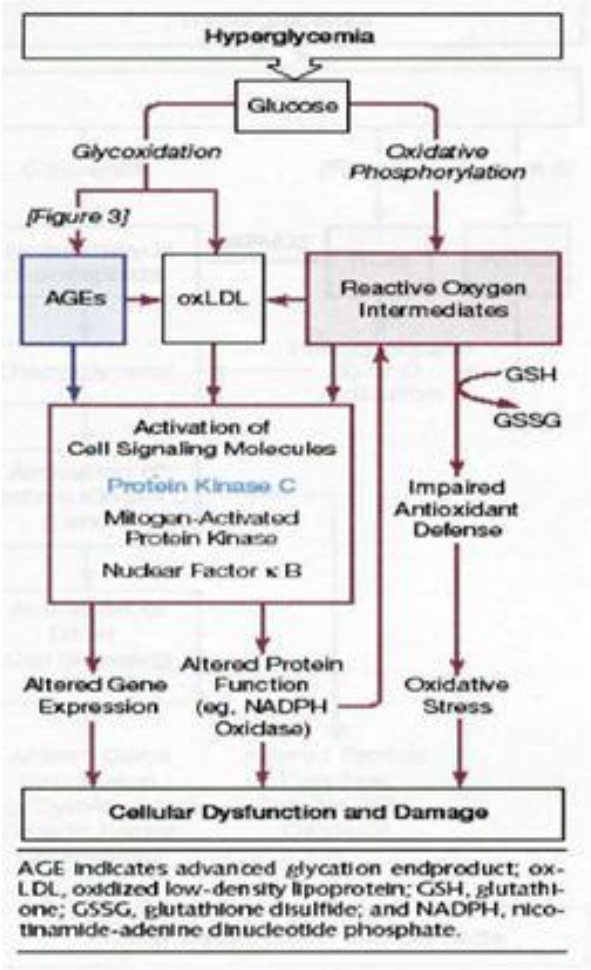


Figure No 4: Reactive oxygen intermediate Pathway

4. Protein kinase theory:

Protein kinase C and diacylglycerol are critical intracellular signaling molecules that can regulate many vascular functions including permeability, vasodilator release, endothelial activation and growth factor signaling. Hyperglycemia causes pathological activation of Protein kinase C in diabetes increasing glycolytic pathway leading to elevation in the levels of intracellular glyceraldehyde-3-phosphate, which in turn stimulate increased denovo synthesis of through glyceraldehyde-3-phosphate.[35] These

chronically elevated levels of diacylglycerol activates Protein kinase C. In addition diacylglycerol - Protein kinase C can indirectly be activated by reactive oxygen intermediates and advanced glycation end products altering gene expression leading to cellular dysfunction and damage.

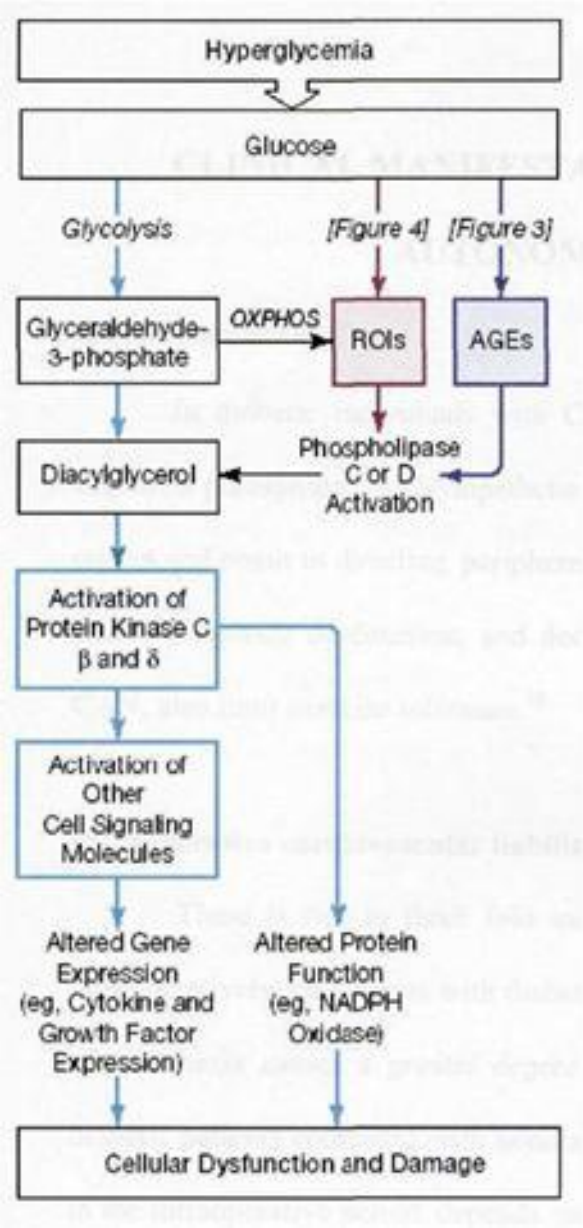


Figure No 5: Protein kinase C Pathway

INVESTIGATIONS FOR DIABETES MELLITUS

Plasma glucose levels:

Glucose is essentially used as a screening parameter. Values are highly diet dependent and drug intake influences the results. Glucose can be estimated chemically and enzymatically. If the fasting plasma glucose > 7.0 mmol/L (126mg/dl) or the random plasma glucose > 11.1 mmol/L (200 mg/dl) then it is considered to be a case of diabetes.

Glucose Tolerance Test (GTT):

The WHO criteria for interpretation of glucose tolerance tests after 75 g oral dextrose ingestion is:[36]

Normal:	Fasting plasma glucose	< 110mg% (< 6.4 mmol/L)
	2hour, plasma glucose	< 140mg%(< 7.8mmol/L)
Diabetes:	Fasting plasma glucose	≥ 126mg% (≥ 7.8 mmol/L) or
	2hour, plasma glucose	≥ 200mg% (≥ 11.1mmol/L)

Impaired glucose tolerance (IGT): any other condition.

A glucose tolerance test in medical practice is the administration of glucose to determine how quickly it is cleared from the blood. The test is usually used to test for diabetes, insulin resistance and sometimes reactive hypoglycemia. The glucose is most often given orally so the common test is technically an oral glucose tolerance test (OGTT).

An abnormal GTT indicates the decreased ability of the body to utilize glucose, thus in turn helping in the diagnosis of diabetes mellitus. An increased glucose tolerance

indicates the ability to utilize more glucose but may be indicative of pathological states like “insulinomas”.

Glycosylated hemoglobin:

Of all the glycosylated form of hemoglobin, HbA_{1c} is the most stable. More than 80 per cent of the glycosylated form is HbA_{1C}. Hence its measurement is taken to be the ideal parameter to understand the "long term diabetic control". This is the most important tool for monitoring diabetes. This test refers to the hemoglobin component formed by interaction with glucose. Since life span of RBC is approximately 120 days, a single HbA_{1c} determination can give information about glycemic control in the preceding 8-12 weeks. The estimation by HPLC method is considered to be gold standard method when compared to other methods. The advantage is that this test does not require any dietary preparations and has low sensitivity but high specificity compared to oral glucose tolerance test.

Complications in diabetes mellitus:

Severe uncontrolled hyperglycemia can lead to

(I) Acute complications

(II) Chronic complications

Acute complication of diabetes:

I) Acute metabolic complications are of two major types,

i) Diabetic ketoacidosis and

ii) Hyperosmolar non ketotic coma.

The former is a complication of Type I, while the latter usually occurs in the setting of Type II.

Diabetic ketoacidosis appears to require insulin deficiency coupled with relative or absolute increase in glucagon concentration. These hormonal changes have two critical effects:

i) They induce maximal gluconeogenesis and impair peripheral utilization of glucose, causing hyperglycemia, which further can induce osmotic diuresis that can lead to dehydration and volume depletion (a Ketoacidotic state)

ii) They also activate ketogenic process and thus initiate the development of metabolic acidosis.

On the other hand hyperosmolar non ketotic diabetic coma which is usually a complication of type II diabetes mellitus results in profound dehydration resulting from sustained hyperglycemia diuresis under circumstances in which the patients is unable to drink enough water to keep up with urinary fluid losses.

Late (Chronic) complications of diabetes:

The diabetic patient is susceptible to a series of complications that cause morbidity and premature mortality. On an average the symptoms develop 15 to 20 yrs after the appearance of overt hyperglycemia. Chronic complications contain 2 types:

I) Nonvascular complications (Gastroparesis, infections & skin changes)

II) Vascular complications are of 2 types

a) Macrovascular complications like circulatory abnormalities (Coronary artery disease, peripheral arterial disease, & cerebrovascular disease)

b) Microvascular complications like retinopathy, nephropathy, neuropathy, diabetic foot ulcers etc.

Circulatory abnormalities:

Atherosclerosis is most extensive complication in diabetes mellitus and occurs earlier than in the general population. The cause of this accelerated course is not known, although, as discussed below, non-enzymatic glycation of lipoprotein may be important. The atherosclerotic lesion appears to be initiated by oxidized LDL, a scavenge receptor. Both HDL and antioxidants have the capacity to impair LDL oxidation, there by exerting an anti-atherogenic action. And the most common source of oxidized LDL is diabetes, which accelerates the oxidative process. Although lipoproteins are often in the normal range, HDL levels tend to be low, while LDL levels are high-normal or high. A low HDL/LDL ratio favors atherosclerosis. Increased levels of Lipoprotein(a) are seen in type I DM but not in type II DM, other important factors are increased platelet adhesions, increased secretion of endothelin-Ic, decreased production of nitric oxide. As a result of these abnormalities coronary artery disease and stroke are more common.

DIABETIC NEPHROPATHY:

Renal disease is a leading cause of death and disabilities in diabetes. About half of the cases of end stage renal disease (ESRD) in the United States are now due to diabetic nephropathy. Approximately 20% - 40% of diabetic patients develop this complication.[21]

Pathophysiology of diabetic nephropathy:

Observational studies have shown that sustained poor glycemic control is associated with a greater risk for development of nephropathy in type 1 and type 2 diabetes.[37] The exact mechanisms for hyperglycemic tissue damage are now elucidated and probably includes:

1) Glycation of proteins leading to the formation of AGEs
2) Over activity of the polyol pathway
3) Generation of reactive oxygen species (ROS)

A key step linking gluco-toxicity to cell dysfunction in diabetic nephropathy is the excess of extra cellular matrix within the glomerulus and interstitium. A number of gene coding matrix proteins in hyperglycemic conditions have been identified.[38] For example, the transcription of the gene transforming growth factor-p (TGF-P) is stimulated by hyperglycemia, AGEs, Angiotensin 2 and ROS.[39] One important consequence of glucose stimulated TGF-P is the up regulation of the insulin dependent GLUT-1 transporter in mesangial cells. Glucose is transported through GLUT-1 and is metabolized through glycolysis. In hyperglycemia, increased de novo synthesis of diacylglycerol results in activation of protein kinase C and mitogen-activated kinases. Activation of these enzymes can lead to stimulation of certain gene, including TGF-P and further through signaling pathways induce expression of extra cellular matrix protein. The formation of AGE also generates ROS, which can activate latent TGF-P.[38] Diabetic nephropathy may be functionally silent for long periods (10-15 years). At onset, the kidneys are usually enlarged and show "super function" (i.e. GFR may be 40% above normal).

The next stage is the appearance of microproteinuria (microalbuminuria), the excretion of albumin in the range of 30-300 mg/dl and when proteniuria is greater than 550mg/dl, degree of leakage is termed macroproteinuria. Once microproteinuria phase begins there is a steady decline in renal function, with GFR falling on an average, by about 1ml/min per month. On an average azotemia begins about 12 yrs after diagnosis of diabetes.

DIABETIC NEUROPATHY

Fifty percent of individuals with long standing type I & type II Diabetes mellitus develop neuropathy. In Diabetic peripheral neuropathy clinical features manifest as- Polyneuropathy, mononeuropathy & autonomic neuropathy.

Diabetic peripheral neuropathy (DPN) is one of the most prevalent and complicated conditions to manage among diabetic patients. About 60% to 70% of people with diabetes have mild to severe forms of nervous system damage; resulting in impaired sensation or pain in the feet or hands, slowed digestion of food in the stomach, carpal tunnel syndrome, precursor for foot ulcers and other nerve problems. Diabetes is the major contributing reason for non-traumatic lower extremity amputations (more than 60% of cases).[40]

The most common form of DPN involves the somatic nervous system; the autonomic nervous system may be affected in some patient. Sensorimotor neuropathy is characterized by symptoms such as burning, shooting, tingling sensations, and allodynia (super-sensitivity or pain from normal stimuli). Autonomic neuropathy can cause gastroparesis, sexual dysfunction, bladder incontinence and cardiovascular damage. The occurrence of DPN is primarily dependent on the duration of diabetes and level of glycemic control. If diagnosed early, neuropathy can be reversed or at least controlled. There are 3 proposed stages of neuropathy: functional (reversible, biochemical alteration in nerve function); structural (may be reversible, loss of structural change in nerve fibers); and nerve death (irreversible, critical decrease in nerve fiber density and neuronal death).[41]

The best way to manage diabetic neuropathy is through primary prevention, management of early symptoms and relief of pain.[42]

DIABETIC RETINOPATHY

Diabetic retinopathy is the most frequent cause of new cases of blindness among adults aged 20-74 years. By the end of the first 2 decades of disease, nearly all patients with type 1 diabetes will have evidence of retinopathy. Nearly 20% of patients with type 2 diabetes will have retinopathy at the time of diagnosis of diabetes.[40]

Up to 90% of blindness due to diabetes is preventable with regular eye examinations and timely treatment. As a general recommendation, all diabetic patients should have annual dilated eye examinations. Early detection of any visual problems is critical. Diabetic retinopathy can progress from mild nonproliferative abnormalities to moderate and severe nonproliferative diabetic retinopathy, and finally, to proliferative diabetic retinopathy.[43]

Nonproliferative retinopathy produces blood vessel changes within the retina: bleeding (hemorrhages), weakened blood vessel walls (microaneurysms), leakage of fluid (edema or exudate) and loss of circulation. It generally does not interfere with vision.[44]

However, if left untreated it can progress to proliferative retinopathy. This is very serious and severe. It occurs when new blood vessels branch out or proliferate in and around the retina. It can cause bleeding into the fluid-filled center of the eye or swelling of the retina and lead to blindness.[43]

The duration of diabetes is probably the strongest predictor for development and progression of retinopathy. However, adequate control of blood glucose, blood pressure

and lipid levels can significantly decrease the progression and morbidity of diabetic retinopathy. For patient requiring treatment, laser photocoagulation is effective at slowing the progression of retinopathy and reducing visual loss. However, it does not restore lost vision. Another option for treatment, vitrectomy, is a complex, high-risk surgical procedure. The technique involves draining the inside of the eye to remove any blood, debris and scar tissue, or to alleviate traction on the retina. Both of these treatments can be effective. Neither treatment can restore lost vision, but they can prevent further eyesight loss.[43]

Classification of Diabetic Retinopathy:

The prestigious Early Treatment Diabetic Retinopathy Study (ETDRS) research group has classified diabetic retinopathy into the following:[45]

Nonproliferative diabetic retinopathy(NPDR)	
Mild NPDR	Micro aneurysms only. Characterized by dot, blot or flame hemorrhage.
Moderate NPDR	More than just micro aneurysms but less than severe NPDR.Characterised by cotton wool spots.
Severe NPDR	Any of the following: >20 intraretinal hemorrhages in each of 4 quadrants Definite venous beading in 2+ quadrants Prominent intraretinal microvascular abnormalities in 1+ quadrant and no signs of proliferative retinopathy.
Proliferative diabetic retinopathy(PDR)	Neovascular (One or more of the following) Vitreous/preretinal hemorrhage.

Table No 3: Classification of Diabetic retinopathy.

Molecular basis of Retinopathy

The retinal changes in patients with diabetes results from 5 fundamental processes[46]

1. The formation of retinal capillary aneurysms.
2. The development of excessive vascular permeability.
3. Vascular occlusion.

4. The proliferation of new blood vessels and accompanying fibrous tissue on the surface of the retina and optic disc.
5. The contraction of these fibro vascular proliferations and the vitreous.

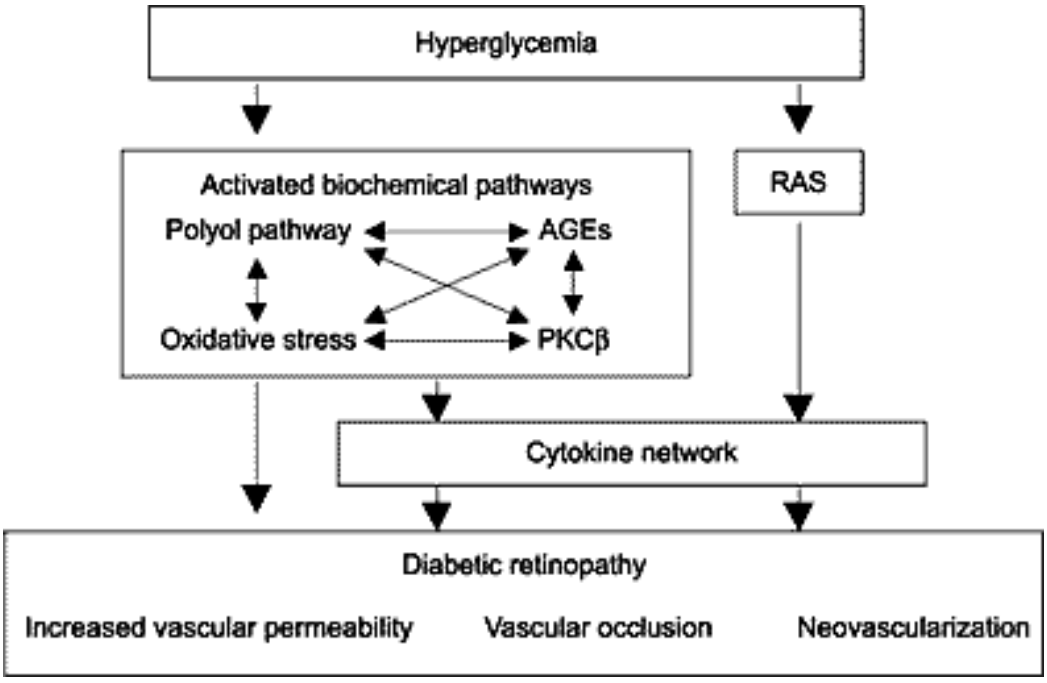


Figure No 6: Pathogenesis of Diabetic retinopathy

Currently 4 major biochemical pathways have been hypothesized to explain the mechanism of diabetic eye diseases, all starting initially from hyperglycemia induced vascular injury.[47] These mainly include:

1. Enhanced glucose flux through the polyol pathway
2. Increased intracellular formation of Advanced Glycation End Products
3. Activation of protein kinase C isoforms
4. Stimulation of Hexosamine pathway

Studies have suggested that these mechanisms seem to reflect a hyperglycemia induced process initiated by superoxide overproduction by the mitochondrial electron transport

chain.[48] Retinal abnormalities are also related to markers of inflammation and endothelial dysfunction, consistent with microvascular pathology. In addition, abnormal autoregulation of blood flow to various tissues and organs has been demonstrated in patients with diabetic Retinopathy.[49]

Risk factors:

It is not possible to define which diabetic individuals will present with retinopathy. However, it is possible to determine the risk factors contributing to the severity of the disease.[2] There are a series of risk factors related to the development and progression of diabetic retinopathy such as duration of diabetes, poor glycemic control, dyslipidemia, hypomagnesemia etc.[2]

Visual disability from diabetes mellitus is a significant public health problem. However the morbidity is largely preventable and treatable. This underscores the need for routine retinal screening of diabetic individuals annually to detect diabetic retinopathy and prevent visual impairment. If managed with timely intervention, the quality of life can be preserved. In addition, optimized control of systemic considerations, which affect onset and progression of diabetic retinopathy through an intensive, multidisciplinary, diabetic retinopathy health care team-based approach, can markedly reduce impairment of vision due to diabetic retinopathy.[50]

Management:

Clinical trials have conclusively shown that good glycemic control substantially reduced the risk of development and progression of diabetic retinopathy. The beneficial

effect of pan retinal photocoagulation in high risk proliferative retinopathy has also been demonstrated, thereby reducing the risk of severe visual loss.[51]

Screening for Diabetic Retinopathy:

Screening of these patients is done by fundoscopic examination every year once diabetes mellitus is diagnosed and more frequent examination of the patients once diabetic retinopathy is diagnosed.[51]

But this is based on fundoscopy, fluroscien angiography and fundus photography which is difficult for clinicians in most of the set up. Beetham W has noted that most useful classification divides retinopathy into only 2 types, proliferative and non-proliferative.[52]This classification would probably be agreed upon most universally among clinicians today because it is based on fundoscopy.

Features of Nonproliferative diabetic retinopathy include microaneurysms, hemorrhages and hard exudates. Microaneurysm, the earliest abnormality, is a small 20-200µm red spot. Hemorrhages are larger, appearing flame shaped if superficial or round if lying deeper in the retina. Hard exudates are shiny, yellowish white opacities. Preproliferative changes i.e., beading, looping or reduplicating of veins, multiple hemorrhages and abundant cotton wool spots indicate worsening ischemia and carry a high risk of progression to neovascularisation.

Proliferative retinopathy is defined by formation of new vessels. New vessels are fine, branching vessels arising from veins often near a bifurcation. Complications of proliferative retinopathy include pre retinal hemorrhage, vitreous haemorrhage and traction causing retinal detachment. Neovascularisation of iris (rubeosis irids) can lead

to neovascular glaucoma. Maculopathy occurs when retinal edema involves the macula and may impair visual acuity.

FREE RADICALS AND REACTIVE OXYGEN SPECIES:

Electrons in atoms occupy regions of space known as orbital. Each orbital can hold a maximum of two electrons. A free radical can be defined as a chemical species possessing an unpaired electron.[53] It can also be considered as a fragment of a molecule. These are conventionally represented by a superscript bold dot: $R^{\bullet}$.

A variety of different living cells make up the human body. These cells are made up of molecules and these molecules are made up of atoms. Electrons travel around the atoms in shells. The inner shell is considered full when it has two electrons; after two, the electrons begin to fill the other shell. The outer shell - and how many electrons are in it is the biggest factor in determining the atom's chemical behavior. Atoms aim to find some sort of stability. For this reason, they try to fill their outer shell by gaining or losing electrons, or bonding with other atoms and sharing electrons. Once this bonding takes place, a molecule is formed. Normally, after bonding, all electrons in a molecule are paired: there is rarely a lone electron by itself. But when this does happen, free radicals form. Desperate and unstable, free radicals do whatever it takes to capture the electron they need to stabilize themselves. This desperation and instability causes free radical to attack nearby molecules. If the molecule under attack loses an electron, it becomes a free radical and repeats the process. A domino effect then begins and the living cell is harmed.

Free radicals can be positively charged, negatively charged or electrically neutral.

As such, free radicals can be formed in three ways.

1) By the homolytic cleavage of a covalent bond of a normal molecule with each fragment retaining one of the paired electrons.
2) By the loss of a single electron from a normal molecule
3) By the addition of a single electron to normal molecule.[8]

Free radical species are unstable and highly reactive. They become stable by acquiring electrons from nucleic acids, lipids, proteins, carbohydrates or any nearby molecule causing a cascade of chain reactions resulting in cellular damage and disease.

Major Types of Free Radicals and Their Derivatives in Living Organism

A. Reactive Oxygen Species

The superoxide anion is formed by the univalent reduction of triplet-state molecular oxygen ($^3O_2$). This process is mediated by enzymes such as NAD(P)H oxidases and xanthine oxidase or nonenzymically by redox-reactive compounds such as the semi-ubiquinone compound of the mitochondrial electron transport chain. SODs convert superoxide enzymically into hydrogen peroxide.[54] In biological tissues superoxide can also be converted nonenzymically into the nonradical species hydrogen peroxide and singlet oxygen ($^1O_2$).[55] In the presence of reduced transition metals (e.g., ferrous or cuprous ions), hydrogen peroxide can be converted into the highly reactive hydroxyl radical ($OH^{\bullet}$). Alternatively, hydrogen peroxide may be converted into water by the enzymes catalase or glutathione peroxidase. In the glutathione peroxidase reaction glutathione is oxidized to glutathione disulfide, which can be converted back to glutathione by glutathione reductase in an NADPH-consuming process.

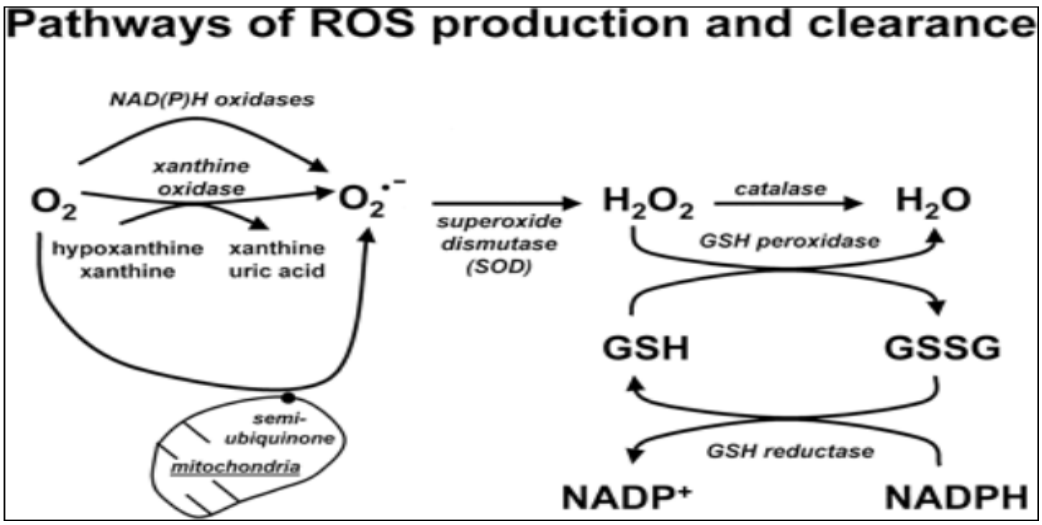


Figure No 7: Pathway of ROS production and clearance

Because superoxide and Nitric oxide (NO) are readily converted by enzymes or nonenzymic chemical reactions into reactive nonradical species such as singlet oxygen ($^1O_2$), hydrogen peroxide, or peroxynitrite ($ONOO^-$), i.e., species which can in turn give rise to new radicals, the regulatory effects of these nonradical species have also been included in this review. Most of the regulatory effects are indeed not directly mediated by superoxide but rather by its reactive oxygen species (ROS) derivatives. Frequently, different reactive species coexist in the reactive environment and make it difficult to identify unequivocally which agent is responsible for a given biological effect. The different types of Reactive Oxygen Species are as follows.[56]

Radicals		Non radicals	
Hydroxyl	$^{\bullet}OH$	Peroxynitrite	$ONOO^{-}$
Alkoxyl	$L(R)O^{\bullet}$	Hypochlorite	^{-}OCl
Hydroperoxyl	$HOO^{\bullet}$	Hydroperoxide	$L(R)OO^{\bullet}$
Peroxyl	$L(R)OO^{\bullet}$	Singlet oxygen	$^{1}\Delta O_2$
Nitric oxide	$NO^{\bullet}$	Hydrogen peroxide	H_2O_2
Superoxide	$O_2^{-\bullet}$		

B. Reactive Nitrogen Species:

The NO radical ($NO^{\bullet}$) is produced in higher organisms by the oxidation of one of the terminal guanido-nitrogen atoms of L-arginine. This process is catalyzed by the enzyme NOS. Depending on the microenvironment, NO can be converted to various other reactive nitrogen species (RNS) such as nitrosonium cation (NO^{+}), nitroxyl anion (NO^{-}) or peroxynitrite ($ONOO^{-}$).[57] Some of the physiological effects may be mediated through the intermediate formation of *S*-nitroso-cysteine or *S*-nitroso-glutathione.[58]

SOURCES OF FREE RADICALS

Respiratory burst:

Respiratory burst is a process by which host defense system cells like neutrophils, monocytes, eosinophils and macrophages protect against harmful organisms.[59] Through NADPH oxidase system, they generate powerful oxidizing agents like, hydrogen peroxide, hypochlorous acid and oxygen radicals such as super oxide $O_2^{-\bullet}$ and hydroxyl radical ($OH^{\bullet}$) are released into phagocytic vacuole to kill internalized bacteria. [60]

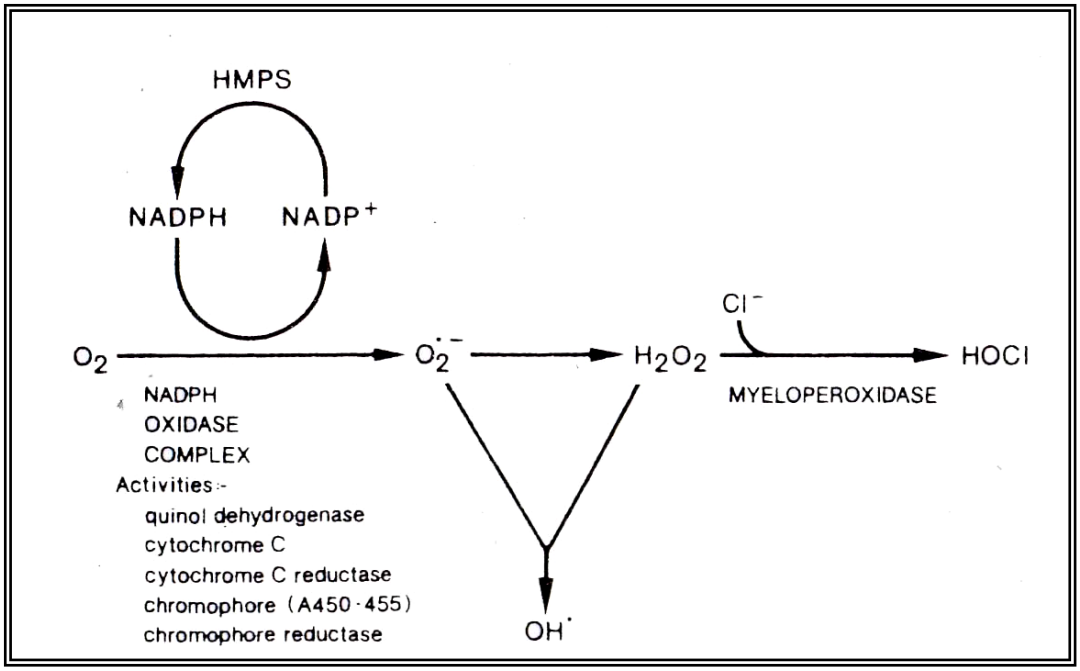


Figure No 8: The production of reactive oxygen species by the NADPH system and myeloperoxidase during the respiratory burst.

Eicosanoid metabolism:

During the formation of the endoperoxide 9,11 endoperoxy 15-hydroperoxy prostaglandin (PGG_2) from arachidonic acid, a trace of hydroperoxide is required to react with the Fe (III) haem at the active site of cyclo-oxygenase enzyme to form a peroxy radical. This reactive oxygen species can stereospecifically abstract a hydrogen atom from arachidonic acid to commence the process of PGG_2 formation. Excess of lipid peroxides can inactivate cyclo-oxygenase activity.[59]

Endoplasmic reticulum and Peroxisome source:

Peroxisomal oxidases and flavo proteins have been shown to produce 35% of liver H_2O_2. D-amino acid oxidase, L-hydroxy acid oxidase and fatty acyl oxidase all produce H_2O_2 but the amount that leaks from the peroxisome into the cytosol is a matter of debate since peroxisomes contain large quantities of the scavenger catalase.[60]

Cytochrome P_{450}/P_{450} reductase and cytochrome b_5 reductase are present in the endoplasmic reticulum and nuclear membranes normally require NADPH and NADH for their reactions but under certain conditions $O_2^{-\bullet}$ and H_2O_2 are released during the catalytic cycle, although the reason for the uncoupling of the reaction from NADP reduction is not well understood.[60]

Mitochondrial sources:

Mitochondria produce $O_2^{-\bullet}$ and H_2O_2 and are probably one of the major intracellular sources of reactive oxygen species. Production of radicals depends largely on the metabolic state of the cell. When the electron transport chain is highly reduced and the respiratory rate is dependent on ADP availability then leakage of electrons from the transport chain is increased and $O_2^{-\bullet}$ and H_2O_2 are produced. This may occur in ischaemic tissue with low O_2 concentration.[60]

Cytosolic sources:

Xanthine oxidase is a cytosolic enzyme normally present in its dehydrogenase form using NAD^+ as its electron acceptor and converting xanthine or hypoxanthine to uric acid. During ischaemia the enzyme is converted to an oxidase form in a Ca^{2+} dependent process. In contrast to the dehydrogenase, the newly formed oxidase uses the molecular oxygen as its electron acceptor and mixtures of $O_2^{-\bullet}$ and H_2O_2 are produced.[60]

Transitional metal in free radical reactions:

Transition metals usually promote free radical reactions. For example, several transitional metal salts reduce H_2O_2 in a one-electron reaction to generate $OH^\bullet$

$$M^{n+} + H_2O_2 \rightarrow M^{(n+1)\bullet} + OH^\bullet + OH^-$$

Where M^{n+} can be (among others) Ti(III), Cu(I), Fe(II), Co(II), or Ni(II). Indeed, chemists have frequently used mixtures of Ti(III) and H_2O_2 as a source of $OH^{\bullet}$ in the laboratory because the reaction is fairly simple.[61]

$$Ti(III) + H_2O_2 \rightarrow Ti(IV) + OH^{\bullet} + OH^-$$

By contrast, the reaction of iron and copper salts with H_2O_2 are by no means simple. For a long time, it was debated as to whether any $OH^{\bullet}$ is formed at all when copper ions react with H_2O_2.[62,63] Only recently has the production of $OH^{\bullet}$ been confirmed by examining the pattern of damage to the Purine and Pyrimidine bases of DNA exposed to copper ions and H_2O_2, and showing that this pattern is characteristic of attack by $OH^{\bullet}$.[64]

The Fenton Reaction:

Several transition metal salts react with H_2O_2 to form $OH^{\bullet}$, but in terms of the possibility of $OH^{\bullet}$ generation in vivo, most attention has been paid to iron,[62,65] although interest in copper is increasing.[63,64.]

Ferrous salts react with H_2O_2 to form $OH^{\bullet}$ by the so called Fenton reaction, which is usually written as

$$Fe\ (II) + H_2O_2 \rightarrow OH^{\bullet} + OH^- + Fe\ (III)$$

In fact, Fenton chemistry is far more complex. Thus the initial product of reaction mentioned above may be an oxo-iron complex, possibly ferryl, that then decomposes to form $OH^{\bullet}$.

$$Fe(II) + H_2O_2 \rightarrow FeOH^{3+}\ (\text{or}\ FeO^{2+}) \rightarrow OH^{\bullet} + Fe\ (III) \qquad (a)$$

Different ligands to the iron (II) may stabilize this intermediate, so that little $OH^{\bullet}$ is formed, whereas others destabilize it. Thus iron-ethylendiamine tetra acetic acid

chelates are good sources of $OH^{\bullet}$ in the presence of H_2O_2,[62] whereas heme rings appear to stabilize ferryl species**.**

The fact that several different reactive species are probably generated when iron salts are mixed with H_2O_2 goes a long way towards explaining the periodic controversies as to whether or not $OH^{\bullet}$ is generated in Fenton reactions. It most certainly is, but other reactive species are generated as well and may sometimes be responsible for the damage caused to biologic molecules.[62]

The above two reactions generate Fe (III). Most ferric complexes react more slowly (if at all) with H_2O_2 than do Fe (II) complex, so that reducing agents stimulate Fenton reaction. This occurs with ascorbate,[62] for example:

$$\text{Fe(III)} + \text{Ascorbate} \rightarrow \text{Fe(II)} + \text{semidehydroascorbate} \qquad \text{(b)}$$

Hence, iron salt-ascorbate- H_2O_2 mixture are good sources of $OH^{\bullet}$ radical; indeed they have been used to generate $OH^{\bullet}$ for determination of reactant rate constants.[66]

Super oxide can reduce certain ferric chelaters. Reaction of Fe(III) with $O_2^{\bullet-}$ appears to proceed via a perferryl intermediate.[62]

$$\text{Fe(III)} + O_2^{\bullet-} \leftrightarrow (\ Fe^{3+}\text{-}\ O_2^{\bullet-} \leftrightarrow Fe^{2+}\text{-}\ O_2^{\bullet-}) \leftrightarrow \text{Fe(II)} + O_2 \qquad \text{(c)}$$

The sum of the (a) and (c) reactions, ignoring the oxo-iron intermediate is

$$O_2^{\bullet-} + H_2O_2 \xrightarrow{\text{Fe}} OH^{\bullet} + OH^{-} + O_2$$

The above reaction is often called as the iron-catalyzed Haber-Weiss reaction or sometimes the super oxide-driven Fenton reaction.

Damaging Reactions of Free Radicals

Lipid peroxidation:

Lipid peroxidation (LPO) reactions are generally free radical driven chain reactions in which one radical can induce the oxidation of a comparatively large number of substrate molecules, which are represented by polyunsaturated fatty acids (PUFA). This type of chain reaction is initiated by the abstraction of a hydrogen atom from a reactive methylene group of a PUFA residue. [56]

Lipid peroxidation proceeds in 3 steps[67]

a) Initiation phase:

This reaction is initiated by a radical ($R^{\bullet}$)

$LH + R^{\bullet} \longrightarrow L^{\bullet} + RH$ – (1)

b) Propogation phase:

Molecular oxygen rapidly adds to the carbon centered radical ($L^{\bullet}$) formed in this process yielding lipid peroxyl radical ($LOO^{\bullet}$).

$L^{\bullet} + O_2 \longrightarrow LOO^{\bullet}$ - (2)

Which can abstract a hydrogen from another PUFA, analogously to Eq (1)

$LH + LOO^{\bullet} \longrightarrow L^{\bullet} + LOOH$ - (3)

c) Termination phase:

Lipid hydroperoxide (LOOH) is the first, comparatively stable, product of the lipid peroxidation reaction. Under conditions where LPO is continuously initiated a termination reaction limits the extent of LPO yielding non radical products (NRP) and destroying two radicals at once.

$LOO^{\bullet} + LOO^{\bullet} \longrightarrow NRP$ - (4)

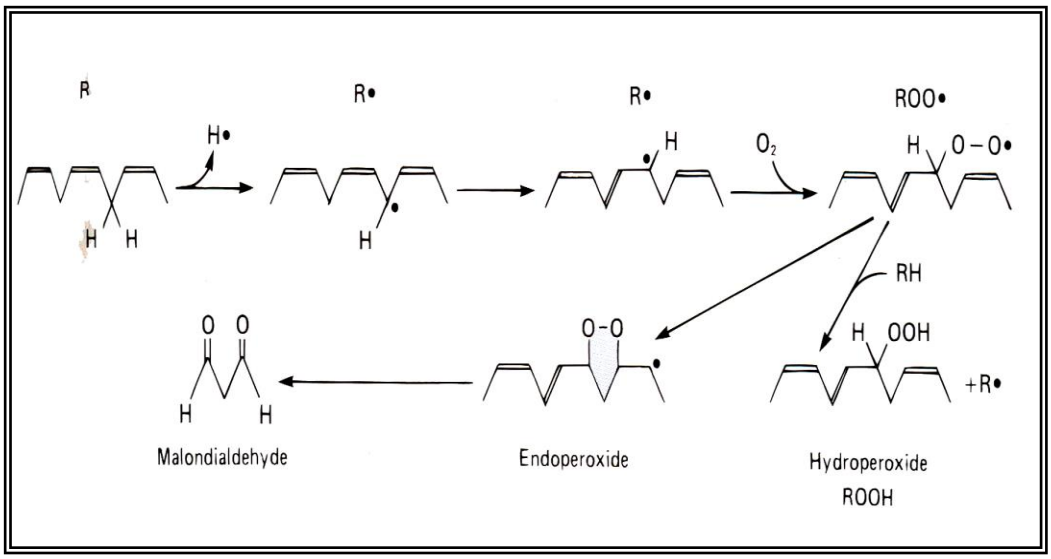


Figure No 9: Lipid peroxidation.

Lipid peroxidation and its role in atherosclerosis:

A crucial step in the pathogenesis of atherosclerosis is believed to be the oxidative modification of low density lipoprotein (LDL). The oxidation of LDL, is a free radical driven lipid peroxidation process and the aldehyde products of lipid hydro peroxide break down are responsible for the modification of the LDL apoprotein. Aldehyde modified apo B protein has altered receptor affinity, causing it to be scavenged by macrophages in an uncontrolled manner with the development of foam cells and the initiation of the atherosclerotic lesion. The aldehyde products of lipid peroxidation may also be involved in other aspects of the development of the lesion. The oxidation of LDL may be prevented by its endogenous antioxidant compounds, most prominent of which is α-tocopherol. Consequently an improved antioxidant status may offer possibilities for the prevention of this major disease.[68]

Oxidation of protein:

Proteins and nucleic acids appear less susceptible than PUFA to free radical attack, in that there seems less possibility of rapid progression of destructive chain reaction. Random attack of radicals on proteins is unlikely to be very damaging unless very extensive. Free radical damage to protein is only likely to be significant to the viability of the cell if the damage is allowed to accumulate, which in most cells is not likely or if the damage is somehow focused on specific sites of a particular protein. As an example, protein and non protein thiol group are readily oxidized by means of free radical and the thiol radical produced may dimerize

$$YSH + X^{\bullet} \rightarrow YS^{\bullet} + XH$$

$$R_1S + R_2S^{\bullet} \rightarrow R_1SSR_2$$

Such types of free radical mediated disturbance of thiol group lead to profound change in enzyme activity. Another way that damage may be focused on specific sites of a particular protein is if the protein binds a transition metal ion at a particular site eg., the binding of copper by a histidine residue. In this case, the reaction of the transition metal with hydrogen peroxide can generate hydroxyl radical that will react at or near the metal binding site: this concept is known as "site – specific" damage.[69] The mechanism of free radical injury on protein is still not clear, but a variety of residue modifications can occur such as formation of peroxides[70] and carboxyls the latter of which may be a useful measure of oxidative damage of protein.

Oxidation of nucleic acids*:*

Oxidizing radicals if they are formed in the vicinity, as has been clearly demonstrated by radiation biologists, readily attacks nucleic acids. As with proteins there appears

little possibility of rapid chain reactions occurring and again much significance is attached to site-specific damage and of high intensity, leading to strand breaks. These damages may elude the repair system before replication occurs, leading to mutations. The detection of oxidized nucleo-bases in human urine has been taken as evidence for a continual oxidative attack on DNA.[71] Even with very high level of efficiency of repair, sufficient damage may accumulate over a life time to lead to mutations and hence cancer.

Oxidation of carbohydrates:

Under excess free radical generation, carbohydrates are one among the major targets by ROS. Auto-oxidation of carbohydrates and Ascorbate yields carboxyl compounds eg glyoxal, arabinosine, methyglyoxal, glycoaldehyde, and dehydrascorbate.[72] The carbonyls are reactive with protein amino groups and initiate the Millard's reaction, which forms Schiff base and eventually advanced glycation end products (AGEs), as glycoxidation products, such as N^{ε}- carboxymethyllysine (CML) and pentosidine, which are frequently found in diabetic atherosclerotic tissue.

Assay of ROS:

Some techniques are available to estimate H_2O_2 production in human patients. Thus H_2O_2 (presumably originating from respiratory tract) can be measured in exhaled air,[73] a recent claim is that H_2O_2 is excreted in human urine [74] may, if validated, lead to a useful assay technique. Some assay are mentioned below

Trapping assay:

The only technique that can "see" free radical directly is Electron Spin Resonance (ESR) spectroscopy, which measures the energy change that occurs as unpaired electrons align in response to an external magnetic field. However, biologically important oxygen radical does not accumulate to high enough concentration to be observable by ESR.[75] Identifying highly reactive radical formed in biologic systems can be achieved by two general approaches, the first of which is trapping. The radical is allowed to react with a trap molecule to give one or more stable products, which are then measured. Mentioning of trapping usually brings to mind the technique of spin trapping[75] in which the radical reacts with a spin trap to form a more stable radical, which is detectable by ESR.

Uric acid oxidation:

In primates (including humans beings), uric acid is an end product of purine metabolism, because an active urate oxidase enzyme in not present.[76] Ames et al [76] proposed that uric acid acts as an antioxidant in vivo. Hence measuring the products of attack of ROS on uric acid might be a potential marker for oxidative damage uniquely applicable to human beings and to other primates

Finger print assay:

One can sometimes implicate ROS as agents of tissue injury by examining the type of chemical change that they produce when they react with biological molecule. For example, attack of $OH^{\bullet}$ on DNA produces a pattern of chemical changes to all four purine and pyrimidine bases that seems characteristic of $OH^{\bullet}$. Other oxygen derived

species either do not attack the DNA bases at all ($O_2^{\bullet}$ and H_2O_2) or they modify only guanine (singlet oxygen). This 'DNA finger printing" approach has been used to show that at least some of the DNA damage that occurs when cells and tissue are subjected to oxidative stress is consistent with ROS formation in the nucleus.[77]

Measurement of end products of lipid peroxidation can also be regarded as a 'finger printing' assay. Measurement of some end products of oxidative damage to protein can also be achieved by the 'protein carbonyl' assay.[69]

Defense against free radicals:

Antioxidant is defined as any substance that, when present at low concentrations compared with that of an oxidizable substrate, significantly delays or inhibits oxidation of that substrate.[78]

Antioxidant defense system:

Antioxidant defense system against oxidative stress is composed of several lines and the antioxidants are classified into four categories based on function.[79]

1) First line of defense is the preventive antioxidants which suppress formation of free radical (Enzymes; glutathione peroxidase, catalase, selenoprotein, transferrin, ferritin, lactoferrin, carotenoids etc).
2) Second line of defense is the radical scavenging antioxidants suppressing chain reaction and/or breaking chain propagation reactions: radical scavenging antioxidants.
3) Third category: repair and denovo antioxidant (some proteolytic enzymes, repair enzymes of DNA etc)

4) A fourth line is an adaptation where the signal for the production and transport of the appropriate antioxidant to the right site.

Antioxidants act as: radical scavenger, hydrogen donors, electron donor, peroxide decomposers, singlet oxygen quencher, enzyme inhibitor, synergist and metal chelating agents. Both enzymatic and non enzymatic antioxidants exist in the intercellular and extracellular environments to detoxify ROS. To provide maximum intracellular protection these scavengers are strategically compartmentalized throughout the cell.

Non - enzymatic antioxidants	**Location**	**Properties**
Vitamin C	Aqueous phase of cell	Acts as free radical scavenger and recycles vitamin E
Vitamin E	Cell membrane	Major chain breaking antioxidant in cell membrane.
Uric acid	End Product of purine metabolism	Scavenger of OH radicals
Glutathione	Non protein thiol in cell	Serves multiple roles in the cellular antioxidant defense
Alpha lipoic acid	Endogenous thiol	Effective in recycling vitamin C may also be an effective glutathione substitute
Carotenoids	Lipid soluble antioxidants,located in membrane tissue	Scavengers of reactive oxygen species, singlet oxygen quencher
Bilirubin	End Product of heme metabolism in blood	Extracellular antioxidant
Ubiquinones	Mitochondria	Reduced form are efficient antioxidants
Metalions sequestration Transferrin, ferritin, lactoferrin		Chelating of metal ions, responsible for Fenton reactions.
Nitric oxide		Free radical scavenger inhibitor of LP.

Enzymatic antioxidants	Location	Properties
Superoxide Dismutase (SOD)	Mitochondria and Cytosol	Dismutase Superoxide radicals
Glutathione Peroxidase (GSH)	Mitochondria and Cytosol	Removes hydrogen peroxide and organic hydrogen peroxide
Catalase (CAT)	Mitochondria and Cytosol	Removes hydrogen peroxide

Table No 4: Antioxidants

Oxidative Stress

In healthy organism, Reactive Oxygen Species (ROS) are detoxified by antioxidants, which bind free radicals without becoming toxic themselves. Therefore antioxidants are the most important barrier against ROS action and Peroxides are the most important oxidative substances that are present in healthy organism in small concentrations.

Under physiological conditions, antioxidants are in excess or at least in equilibrium compared to ROS and free radicals. A pathogenic excess of oxidants compared to antioxidants is called Oxidative Stress (OS). Such conditions are caused by enhanced production of ROS or deficit of antioxidants. Consequently the main sign of Oxidative Stress is the lack of protection of the organism to free radical/ROS attacks.

Under normal physiological conditions, approximately 0.1%–5% of oxygen that enters the electron transport chain is reduced to superoxide; a reactive oxygen species (ROS) and the rest are used in metabolic processes. ROS can also be generated from other sources other than the mitochondrial electron transport chain including, cytochrome P450, the NAD(P)H oxidase(s) and nitric oxide synthases.[80]

ROS are produced continuously in all cells to support normal cellular functions. However, excess production of ROS originating from endogenous or exogenous sources, or inefficient removal of ROS, could result in pathological conditions. ROS produced during normal oxidative metabolism are eliminated by an efficient scavenging system, but an imbalance between production and scavenging of ROS can result in excessive levels of either molecular oxygen or ROS, thus resulting in increased "oxidative stress." Hence, oxidative stress is the cytopathic consequence of the generation of excess ROS beyond the capacity of a cell to defend against them and represents an imbalance between excess formation and / or impaired removal of ROS. Consequences of chronic oxidative stress include damage to biological macromolecules such as DNA, lipids, proteins and carbohydrates disruption in cellular homeostasis and generation of other ROS creating further damage resulting in many disease processes of clinical interest. [81]

Oxidative Stress in human diseases:

Apart from its deleterious effect on liver, oxidative stress has been implicated in a large number of human diseases. Oxidative stress contributes to tissue injury following irradiation and hyperoxia. It is suspected to be important in neurodegenerative diseases including Motor Neuron disease, Parkinson's disease, Alzheimer's disease and Huntington's disease. Oxidative stress is thought to be linked to certain cardiovascular disease, since oxidation of LDL in the vascular endothelium is a precursor to plaque formation. Oxidative stress also plays a role in the ischemic cascade due to oxygen reperfusion injury following hypoxia. This cascade includes both strokes and heart attacks. Oxidative stress is also implicated in various chronic inflammatory diseases

(e.g. Rheumatic arthritis, chronic glomerular nephritis, ulcerative colitis etc.) and acute inflammatory disease, where respiratory burst and NAD(P)H oxidase active source of free radical generation. Oxidative stress is also involved in a range of respiratory disease, to mention a few, cigarette smoke contains free radicals, hence bronchitis; Adult Respiratory Distress Syndrome (ARDS), where increased neutrophil activity can induce enhanced ROS generation.[53]

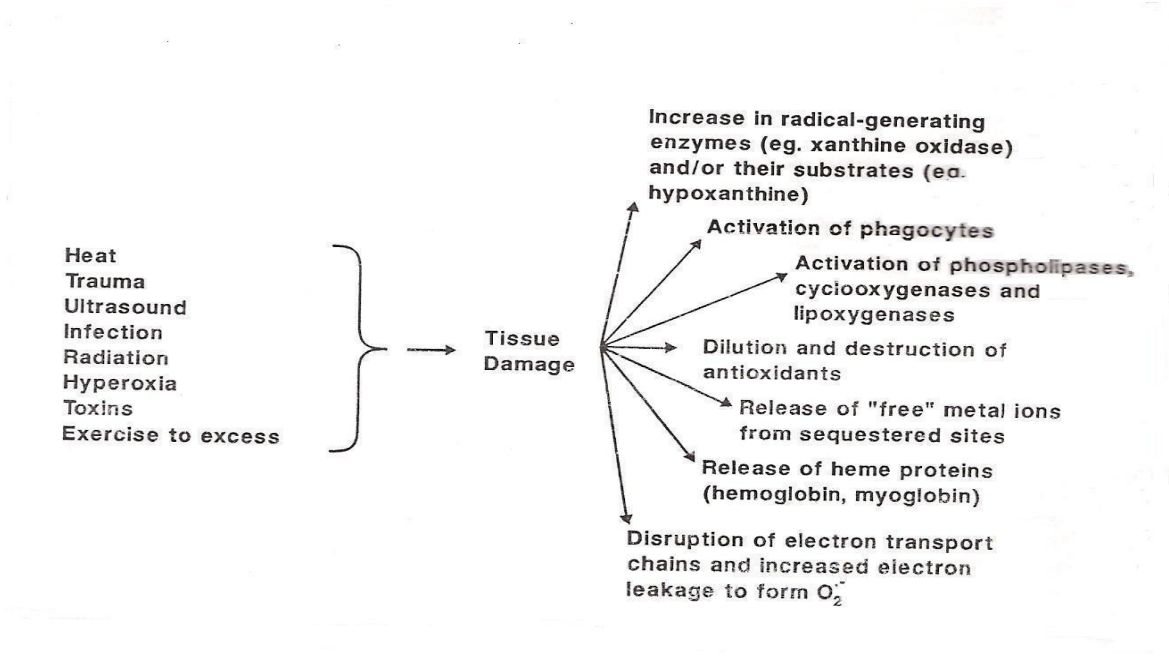


Figure No 10: Tissue Damage in turn leading to oxidative stress

Oxidative stress markers:

Oxidants are highly reactive compounds with a half-life of only seconds. Therefore there in vivo determination is generally not feasible. In contrast lipids, proteins, carbohydrates and nucleic acids after being modified by oxygen radicals have lifetimes ranging from hours to weeks, which make them ideal markers of oxidant stress.

Markers of oxidative stress:

1) Lipid peroxidation
    a. Acrolein
    b. Malonedialdehyde
    c. 4-hydroxynonenal
    d. Thiobarbituric acid reactive substance
    e. F_2-isoprotenes
    f. Advanced Lipoxidation End Products (ALE)
    g. Oxidized LDL antibodies
2) Advance oxidation protein products
3) Advanced glycosylation end products
4) Enzymatic markers
    a. Super oxide dismutase
    b. Glutathione peroxidase
    c. Catalase
    d. Glutathione reductase
5) Non- enzymatic
    a. Glutathione
    b. Vitamin E
    c. Vitamin C
    d. Ferritin
    e. Transferrin
    f. Albumin etc.

Oxidative stress in diabetes:

Diabetes results in increased oxidative stress and elevated oxidative stress plays an important role in the pathogenesis of diabetic complications.[82]Increased oxidative stress in diabetes is postulated to promote the development of neuropathy, nephropathy, myocardial injury and retinopathy.[83]

The possible sources of oxidative stress in diabetes might include autooxidation of glucose, shifts in redox balances, decreased tissue concentrations of low molecular weight antioxidants such as reduced glutathione (GSH) and vitamin E and impaired activities of antioxidant defense enzymes such as superoxide dismutase (SOD) and catalase.[84]

ROS generated by high glucose are considered as a causal link between elevated glucose and the other metabolic abnormalities important in the development of diabetic complications.[85]

Oxidative stress and diabetic retinopathy

The retina has high content of polyunsaturated fatty acids and has the highest oxygen uptake and glucose oxidation relative to any other tissue. This phenomenon renders retina more susceptible to oxidative stress.[86] It has been suggested that the correlation between hyperglycemia, changes in the redox homeostasis and oxidative stress are the key events in the pathogenesis of diabetic retinopathy.

Since oxidative stress represents an imbalance between excess formation and/or impaired removal of ROS, the antioxidant defense system of the cell is a crucial part of the overall oxidative stress experienced by a cell. In diabetes, the activities of

antioxidant defense enzymes responsible for scavenging free radicals and maintaining redox homeostasis such as SOD, glutathione reductase, glutathione peroxidase and catalase are diminished in the retina.[84]

Further, the cell is equipped with intracellular antioxidant, GSH; GSH is probably the most important defense the cell is equipped with. It can act as an ROS scavenger and modulate intracellular redox state. The levels of this intracellular antioxidant are decreased in the retina in diabetes and the enzymes responsible for its metabolism are compromised.[87] Apart from the antioxidant defense enzymes, nonenzymic antioxidants such as vitamin C, vitamin E, and β-carotene that exist biologically for the regulation of redox homeostasis are also depressed during hyperglycemia induced oxidative stress.[88]

Glutathione (GSH):

Glutathione is a sulfhydryl containing tripeptide made up of glycine, glutamic acid and cysteine[14] GSH was first described as philothione, 100 years ago and it comprises the bulk of the pool of free thiol groups in biological system. It functions as mediators of many physiological and pathophysiological processes in detoxification. GSH is a co-substrate in the GSH-peroxidase mediated reaction for the reduction of hydro peroxides in the defense against oxidative stress, in protein and DNA synthesis and alone, as with other Thiols, in protection of DNA from damage resulting from ionizing radiation. It also assumes a pivotal role in numerous cellular functions including bio reduction reaction, maintenance of enzyme activity, amino acid transport and protection from harmful oxidative species and detoxification of xenobiotic. GSH levels in various tissues like liver, lungs, brain etc. parallels its levels in blood and in the blood almost all of the reduced glutathione is found within erythrocyte.[89] The intracellular levels of GSH

in mammalian cells are in milli-mole range (0.5 to10 mM), whereas micro molar concentration is typically found in plasma. GSH is also a major intracellular non-protein bound sulphydryl molecule.

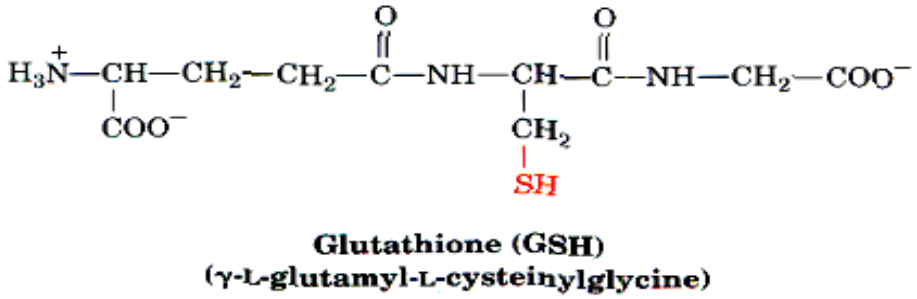


Figure No 11: Structure of Glutathione.

Synthesis of Glutathione:

GSH is a tripeptide derived from glycine, glutamate and cysteine. The first step in synthesis is a condensation of the gamma carboxyl group of glutamate with the alpha amino group of cysteine. The carboxyl group is first activated by ATP to form an acyl phosphate intermediate, which is then attacked by the cysteine amine group. The second step is similar, with the alpha carboxyl group of cysteine activated to an acyl phosphate to permit condensation with glycine. Glutathione is virtually present in all the cells, often at high levels and can be thought of as a kind of redox buffer. It probably helps maintain the sulfhydryl groups of proteins in the reduced state and the iron of heme in the ferrous (Fe^{2+}) state and it serves as a reducing agent for glutaredoxin.[14] Its redox function can also be used in removing toxic peroxides that forms in the course of growth and metabolism under aerobic conditions:

$$2GSH + R\text{-}OOH \rightarrow GSSG + H_2O + R\text{-}OH$$

This reaction is catalyzed by Glutathione peroxidase, a remarkable enzyme, in that it contains a covalently bound selenium (Se) atom in the form of Selenocysteine. The selenium is essential for the enzyme's activity. The oxidized form of Glutathione (GS-SG) contains two molecule of glutathione linked by a disulfide bond.

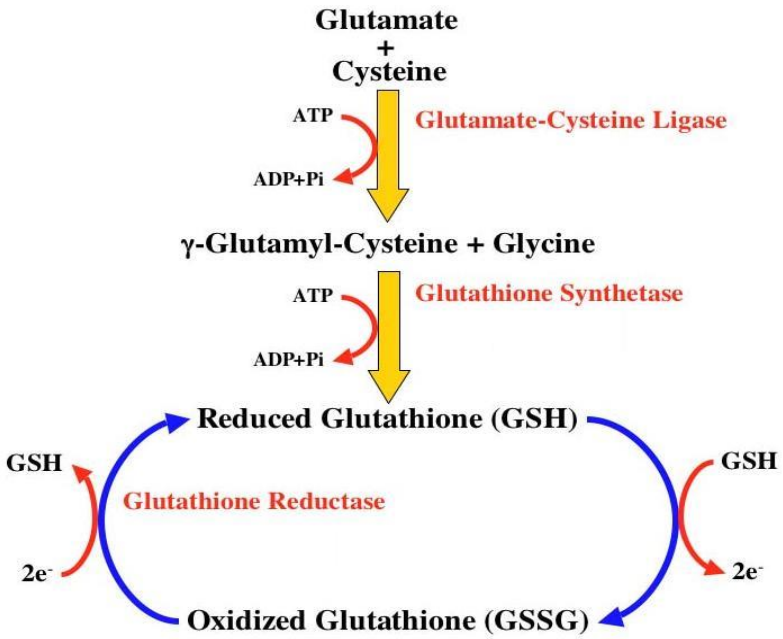


Figure No 12: **Synthesis of Glutathione**

L-Ascorbic Acid (Vitamin C):

Ascorbic Acid is a water soluble vitamin serves as reducing agent in several important hydroxylation reactions in the body. Vitamin C present in most plant foods especially citrus fruits, berries, melons, tomatoes, green peppers, broccoli, brussels sprouts, potatoes and green leafy vegetables.[15] Dietary deficiency leads to scurvy. It is now known that the vitamin is an organic acid whose properties are related to its facility to be oxidized reversibly to dehydroascorbic acid. Its physiological functions are varied and probably not fully worked out. It is generally believed that its oxidation-reduction system plays an important role in biological oxidations and reductions and in cellular

respiration. It is involved in tyrosine metabolism. It is required for the conversion of folic acid to folinic acid. It is also required for normal erythropoiesis. Chronic Vitamin C deficiency has an impaired conversion of cholesterol to bile acids. Thus the effect of deficiency of the vitamin may be of considerable importance even although signs of scurvy are absent.

The normal plasma ascorbic acid level varies between 0.7 – 1.2 mg/ 100 ml of plasma.

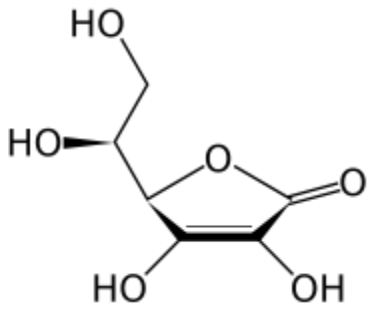


Figure No 13: Structure of Ascorbic Acid:

Deficiency of Ascorbic Acid:

Gross deficiency of vitamin C results in Scurvy.[15]It is manifested by hemorrhagic tendencies- petechiae in mild deficiency and as ecchymoses or even hematoma in severe conditions; painful, swollen and spongy gum; weak scorbutic bones which fractures easily; microcytic, hypochromic anemia or die suddenly from heart failure.[90]

Diseases caused by Vitamin C deficiency that might reflect its role as an antioxidant include an increased risk of coronary heart disease, as demonstrated in a cohort of Finnish men[91]and an increased risk of death by stroke in a cohort of elderly British people.

GLYCATED HEMOGLOBIN

Glycation is the nonenzymatic addition of a sugar residue to the amino group of proteins. Human adult hemoglobin usually consists of HbA (97%), HbA_2 (2.5%) and HbF (0.5%). Chromatographic analysis of HbA identifies several minor hemoglobins namely HbA_{1a}, HbA_{1b} and HbA_{1c} which are referred to as glycated hemoglobins.[12]

HbA_{1c} is the major fraction, constituting approximately 80% of HbA_1.It is formed by the condensation of glucose with the N-terminal valine residue of each β-chain of HbA to form an unstable Schiff base (aldimine). The schiff base may either dissociate or undergo an amadori rearrangement to form a stable ketoamine.

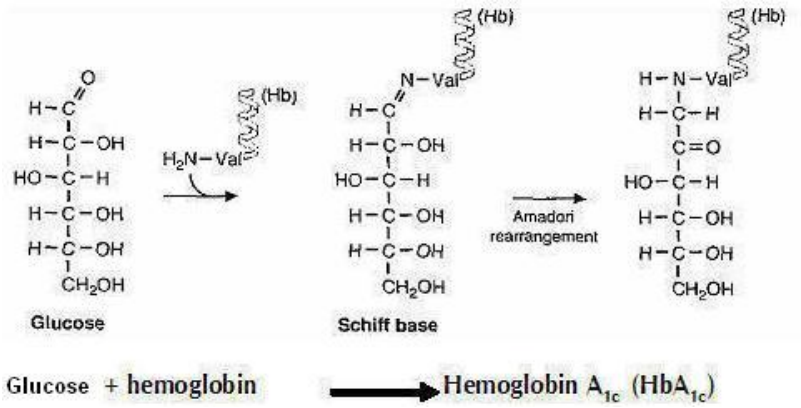


Figure No 14: **Formation of HbA_{1c}**

Formation of glycated hemoglobin (GHb) is essentially irreversible and the concentration in the blood depends on both the life span of the red blood cells (average 120 days) and the blood glucose concentration. Measurement of glycated hemoglobin is effective in monitoring long term glucose control in people with diabetes melitus. It provides a retrospective index of the integrated plasma glucose values over the preceding 6-8 weeks and is not subject to the wide fluctuations observed when assaying

blood glucose concentrations. In addition glycated hemoglobin is a measure of the risk for the development of complications of diabetes mellitus.[12]

HbA_{1c} amounts to 5-8% of total HbA_1 in healthy individuals and ranges from 8-30% in patients with diabetes mellitus, depending on the degree of control of blood glucose concentration. In conditions of sustained hyperglycemia the proportion of hemoglobin that is glycated is increased substantially.[12] Studies have shown a significant positive correlation between HbA_{1c} as well as duration of diabetes mellitus.[92]

Non-enzymatic glycation results in the formation of covalent connections between the aldehyde group of glucose and free amino groups of proteins. The ultimate products of the reaction are advanced glycation end products (AGEs). Changes in the structure of collagen and intercellular matrix due to advanced glycation end products formation influence the properties of connective tissues. Advanced glycation end products also react with macrophage receptors, releasing cytokines and growth factors and thus stimulate destructive and mitogenic processes.[93]

Moreover, the synthesis of advanced glycation end products is a source of free radicals. The toxic oxygen derivatives inhibit nitric oxide synthase and this reaction is the source of next pool of free radicals. Reduced availability of a vasodilator such as nitric oxide could contribute to the reduced blood flow through the small blood vessels.[94]

The role of glyoxidation in the development and progression of diabetic retinopathy has been well documented. Results of Diabetes Control and Clinical Trial (DCCT) strongly supported the causal role of chronic hyperglycemia in the pathogenesis of diabetic

microvascular complications. The risk of diabetic retinopathy increases stepwise with increasing degrees of hyperglycemia.[95]

Many epidemiological studies have demonstrated a strong relation between hyperglycemia and the development and progression of diabetic retinopathy. Elevated HbA_{1c} levels have been found to be a potent predictor of progression of proliferative diabetic retinopathy.[96] The interaction of advanced glycation end products and their receptors and increased activity of the polyol pathway have been implicated as mediators of increased microvascular permeability, ischemia and angiogenesis.

HbA_{lc} as a screening test for diabetes:

There remains considerable interest in extending the use of glycosylated hemoglobin measurement to include the diagnosis besides the monitoring of diabetes. Most studies have brought out the fact that glucose tolerance test (GTT) is a more sensitive diagnostic method and impaired GTT may occur with normal glycosylated hemoglobin (GHb).[97] Studies have found HbA_{lc} to be of limited value as a screening test because of the large number of subjects who have either impaired glucose tolerance or frank diabetes, but have HbA_{lc} values that are within the non-diabetic reference interval. Thus, a raised HbA_{lc} would appear to be specific for diagnosing diabetes but the test is not particularly sensitive.

Certain studies have shown HbA_{lc} to be a good predictor of microvascular disease compared to fasting or two-hour post-OGTT glucose value.[98]It has been demonstrated that HbA_{lc} can be high with normal GTT and high values can occur in

non-diabetics. A genetic polymorphism has been described which influences the rate of glycosylation but probably the prevalence of such polymorphism is low.

In conclusion, within the past ten years, studies using HbA_{1c} have answered positively the fundamental question as to whether glycemic control influences the outcome of patients with diabetes. Therefore, despite its inherent limitations, HbA_{1c} seems destined to continue to be the most valuable parameter for assessing glycemic control.

Methods of the determination of Glycated Hemoglobin:

Methods to separate hemoglobin from glycated hemoglobin using the following techniques based on:[12]

1. Charge differences [Ion exchange chromatography, High Performance Liquid Chromatography (HPLC), Electrophoresis, Isoelectric focusing]
2. Structural differences (Affinity chromatography and Immunoassay)
3. Chemical analysis (Photometry and Spectrophotometry)

Result of Glycated Hemoglobin is expressed as a percentage of total hemoglobin.

LIPIDS AND DIABETIC RETINOPATHY:

Of many metabolic derangements caused by chronic uncontrolled diabetes mellitus, the lipid status requires a special attention, due to its clinically significant correlation with various cardiovascular accidents.

General over view of lipoprotein:

Lipoproteins are a biochemical assembly that contains both proteins and lipids that may be structural or catalytic in function. Lipoprotein may be enzymes, proton pumps, ion pumps for transport, or some combination of these functions. However, lipoproteins function as carrier of fat still stands out as the most important function. The protein particles have charged group aimed outward so as to attract water molecule this makes them soluble in the salt-water bared blood pool. Triglycerides and cholesterol is added to or removed from the lipoprotein transport particles.[13]

Lipoproteins, classified based on their density are listed in order from larger and less dense (more fat than protein) to smaller and more dense (more protein less fat).

1. Chylomicrons carries triacylglycerol from the intestine to the liver and to adipose tissue.
2. Very Low Density Lipoprotein (VLDL) carries newly synthesized triacylglycerol from liver to adipose tissue
3. Intermediate density lipoproteins (IDL) are intermediate between VLDL and LDL. They are not usually detectable in the blood.
4. Low density lipoprotein (LDL) carry cholesterol from liver to cells of the body sometimes referred to as "bad cholesterol" lipoprotein.
5. High density lipoprotein collects cholesterol from the body's tissue and brings it back to the liver. Sometimes referred to as "good cholesterol" lipoprotein.
6. Lipoprotein (a)

Apart from the above classification they can also be classified as alpha and beta based on serum proteins electrophoretic movement.

Lipid status in diabetic mellitus:

Hyper lipidemia, hyper lipoprotienemia or dyslipidemia is the presence of elevated abnormal levels of lipids and/or lipoprotein in the blood. Diabetes mellitus is one of the commonest causes of such derangements in the lipid status, however, some patients exhibit hyper lipidemia even when diabetic control is adequate, these patients likely have a primary familial hyperlipoprotenemia that is independent of diabetes. Hypertriglycerdemia, which denote high blood levels of triglycerides, the most abundant fatty molecule is common in diabetics and is due to both, over production of VLDL in the liver and to a disposal defect in the periphery. The latter is a consequence of a deficiency of lipoprotein lipase an insulin dependent enzyme. Of these abnormalities individually considering hypercholesteremia may be worthwhile.[13]

Hypercholesteremia is the presence of high level of cholesterol in the blood. It is a metabolic derangement that can be secondary to diabetes and many other diseases and can contribute to many form of disease, most notably cardiovascular disease. It is closely related to the terms hyperlipidemia and hyperlipoprotenemia. When measuring cholesterol, it is important to measure its sub fraction before drawing a conclusion as the cause of the problem. Among the sub fractions LDL, HDL & VLDL are considered. In the past LDL and VLDL levels were rarely measured directly, due to cost concerns. VLDL levels are reflected in the levels of triglycerides, where as LDL was usually estimated as a calculated value from other fraction this method is called the Friedewald calculation: LDL = total cholesterol - (HDL + TG/5).

There are increasing evidence that chronic uncontrolled diabetes increases the level of triglycerides in the blood, lowers HDL and increase in LDL particle distribution.

Altered levels are associated with atherosclerosis, myocardial infarction, shock and peripheral vascular disease. This is why cholesterols inside LDL lipoprotein is called "bad cholesterol". Increasing evidence has revealed that the concentration and size of LDL particles, more powerfully relates to the degree of atherosclerosis progression than the concentration of cholesterols contained with all the LDL particles. In a healthy person ideally small number of large LDL particles correlate with much faster growth of atheromas, hence progression of atherosclerosis. LDL is formed as VLDL lipoproteins, which lose triglycerides through the action of lipoprotein lipase (LPL) and becomes smaller and dense, containing a higher proportion of cholesterol. LDL poses maximum risk for cardiovascular disease when it invades the endothelium and becomes oxidized. A complex act of biochemical reaction regulates the oxidization of LDL, chiefly stimulated by presence of free radicals in the endothelin.

Apart from cholesterol concentration and associated transport systems, triglycerides, which are also increased to greater extent, is observed in diabetes. High levels of triglycerides is linked to atherosclerosis and by extension, risk of heart disease and stroke. However the negative impact of raised levels of triglycerides is lower than that of LDL: HDL ratio. The risk can be partly accounted to a strong inverse relationship between triglycerides levels and HDL-cholesterol levels.

In type 2 diabetic patients, quantitative and qualitative abnormalities in lipoproteins are presumed to be responsible for the increased risk of vascular diseases. Each lipid and lipoprotein fraction is affected by insulin resistance and hyperglycemia.

Patients with type 2 diabetes mellitus typically have lipid profile characterized by elevated triglyceride, low HDL, modestly elevated LDL, elevated levels of Lp(a) and hypercholesterolemia. In addition studies over the years have suggested a relationship between serum lipid levels and diabetic retinopathy.[99]

Ishrat Kareem et.al., showed that there is significant increase in serum cholesterol and triglyceride levels in patients with diabetic retinopathy.[100]

Lill-Inger Larson et.al., found statistically significant association between higher Lp(a) levels, higher triglyceride, higher cholesterol levels and lower HDL-cholesterol /Total cholesterol ratios with severity of retinopathy.[101]

Several other studies have shown conflicting results. Sinav et.al., reported that while total cholesterol, HDL-C and LDL-C were related to proliferative retinopathy, serum triglyceride was not.[102]

Another study showed that cholesterol and triglyceride levels were significantly associated with both the occurrence of any retinopathy as well as the occurrence of mild-severe non proliferative retinopathy and proliferative retinopathy.

The mechanism by which serum lipids may cause progression of diabetic retinopathy is not clearly understood. It has been postulated that elevation of blood viscosity and alterations in the fibrinolytic system occur in hyperlipidemia causing hard exudates formation. There may be incorporation of triglycerides into the cell membrane leading to changes in membrane fluidity and leakage of plasma constituents into the retina. This results in haemorrhage and oedema in the retina.[103]

In a prospective study to investigate the influence of serum lipids on the visual outcome of patients after central laser photocoagulation, Kremser et.al., found that the patients with normal total cholesterol, LDL-C, HDL-C and triglyceride levels tend to have better results than those with abnormal lipid levels.[104]

Sinav found significantly higher levels of HDL, LDL in total serum cholesterol in patients with diabetic retinopathy.[102]A similar association was found in Dornan's study, in which there was no difference in triglyceride levels between patients with proliferative retinopathy and the ones with normal fundus.[105]

Zélia Maria Da Silva Corrêa et al showed, total serum cholesterol appears as an important risk factor, unlike other studies that found only LDL, HDL and triglycerides to be more important risk factors for incidence and progression of diabetic retinopathy.[102, 105]

L. Doman study suggests that raised levels of LDL cholesterol or a reduced ratio of HDL to LDL cholesterol, might play a part in the pathogenesis of diabetic retinopathy. However, proof could only come from a prospective study since disturbed lipid metabolism might be a consequence rather than a cause of microangiopathy. The minor clinical differences are unlikely to have influenced the results significantly since neither diabetes duration, bodyweight nor blood pressure correlated significantly with cholesterol concentrations. However, LDL cholesterol remained significantly correlated with retinopathy.It seems improbable that the association between cholesterol and retinopathy was secondary to the minor differences in diabetic control. poor diabetic control is associated more with hypertriglyceridaemia and hypercholesterolaemia.[106]

The relationship between plasma lipids and retinopathy has practical importance since lipid-lowering agents might be beneficial for retinopathy. Most clinical trials studied patients with severe exudative retinopathy and showed no effect on visual prognosis. One report suggested that a diet rich in polyunsaturated fats delayed the progression of retinopathy.[107]

RESULTS

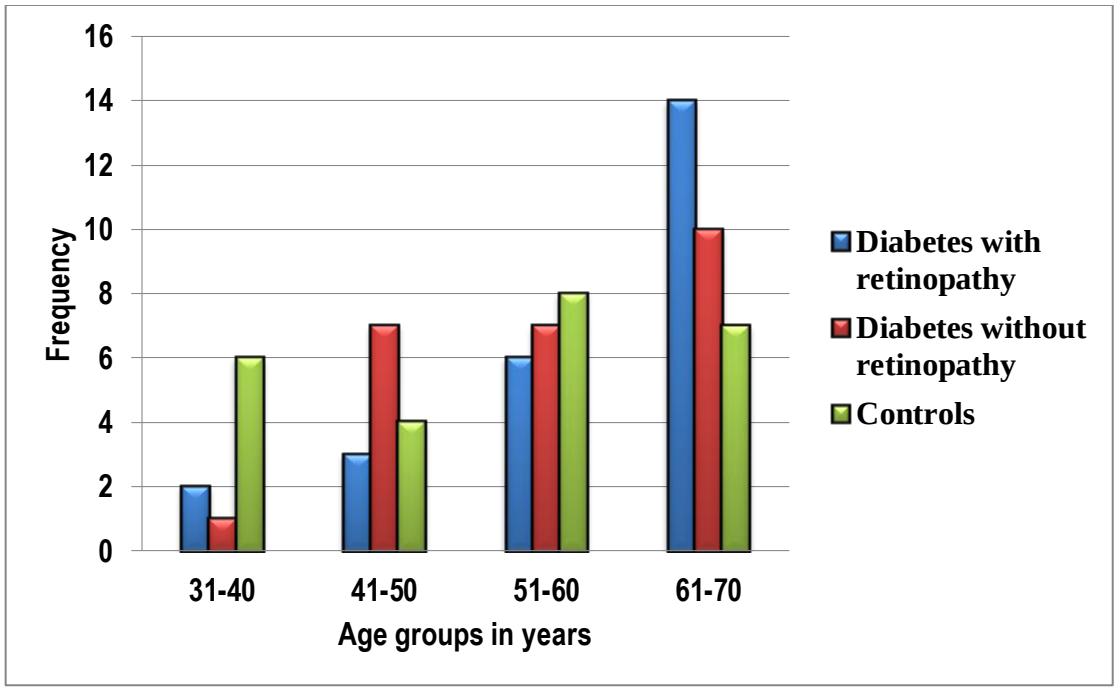
The present study analyses, the correlation between, oxidative stress parameters (Erythrocyte Glutathione, Vitamin C), HbA_{1c}, lipid profile, in diabetic patients with retinopathy and in diabetic patients without retinopathy. These results were compared with the controls.

The study was conducted on 25 diabetic patients with retinopathy, 25 diabetic patients without retinopathy, and 25 controls. The cases and controls were age and sex matched.

The age group was between 30-70 years. The mean age in diabetic patients with retinopathy was 59.52 ± 9.89 yrs, in diabetic patients without retinopathy it was 58.20 ± 10.79 yrs and in controls, it was 51.56 ± 10.92 yrs.

Table No 5: Age distribution in the study groups

Age in Yrs	Diabetic with retinopathy (%)	Diabetic without retinopathy (%)	Controls (%)
31-40	2 (8)	1 (4)	6 (24)
41-50	3 (12)	7 (28)	4 (16)
51-60	6 (24)	7 (28)	8 (32)
61-70	14 (56)	10 (40)	7 (28)
Total	25 (100)	25 (100)	25 (100)



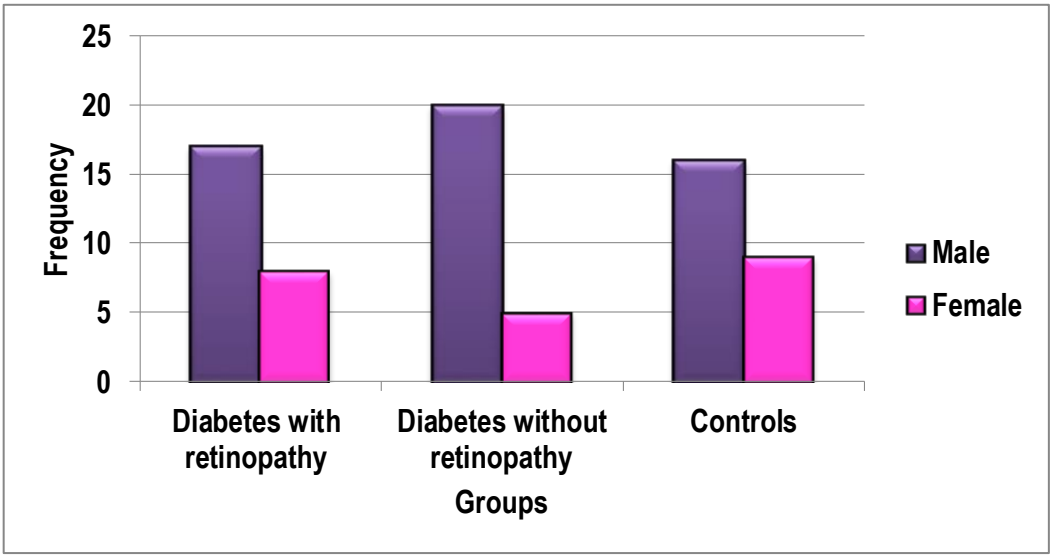
Graph No 3: Bar diagram showing age distribution in the study groups

Table-5 and Graph- 3 shows the distribution of the subjects in different age groups. The cases and controls were age matched.

Table No 6: Gender distribution in the study groups

Gender	**Diabetic with retinopathy (%)**	**Diabetic without retinopathy (%)**	**Controls (%)**
Males	17 (68)	20 (80)	16 (64)
Females	8 (32)	5 (20)	9 (36)
Total	25 (100)	25 (100)	25 (100)

As represented in the above Table No 6, among the diabetic patients with retinopathy, the number of males were 17 and females were 8. Among the diabetic patients without retinopathy, the number of males were 20 and females were 5. The control group included 16 males and 9 females. The cases and controls were sex matched.



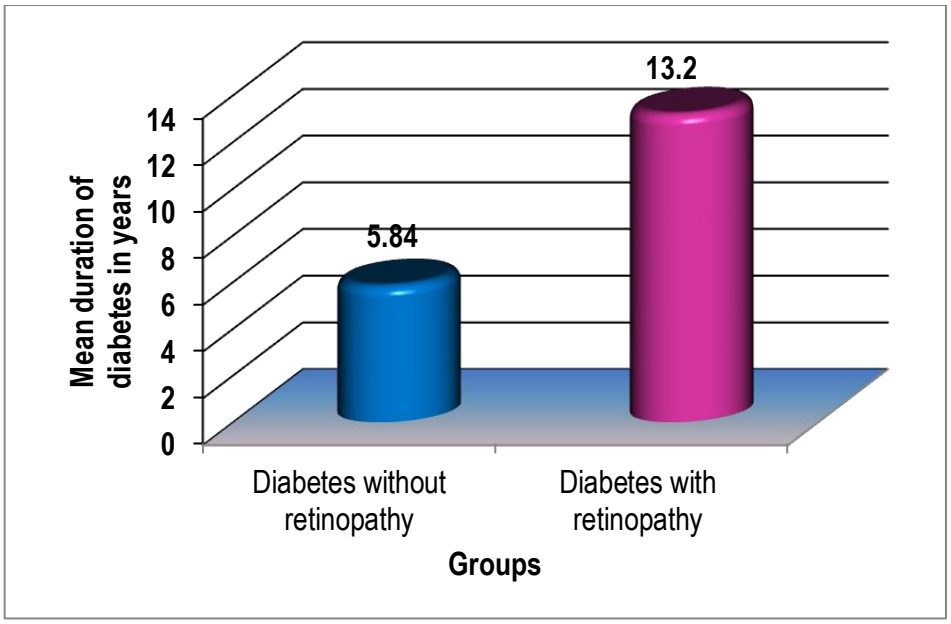
Graph No 4: Bar diagram showing gender distribution in the study groups

Above Table No 6 and Graph 4 shows the gender distribution in the study groups. The number of males who presented with diabetes mellitus with retinopathy and without retinopathy was higher when compared to the females. The above findings show that the incidence of diabetic retinopathy was higher in males when compared to females.

Table No 7: Duration of diabetes mellitus in the study group.

Groups	Mean ±S.D (yrs)	Minimum	Maximum
DM with retinopathy	13.2 ± 1.66	10 yrs	16 yrs
DM without retinopathy	5.84 ± 1.65	2 yrs	8 yrs

The mean duration of diabetes mellitus in diabetic patients with retinopathy as indicated by their history was 13.2 ± 1.66 yrs and in diabetic patients without retinopathy it was 5.84 ± 1.65 yrs.



Graph No 5: Duration of diabetes mellitus in the study groups

The duration of diabetes mellitus in diabetic patients with retinopathy was significantly higher when compared to the diabetic patients without retinopathy ($P < 0.001$). The above findings show that as the duration of diabetes mellitus increases, there is increase in the incidence and progression of retinopathy.

Table No 8: FBS (mg/dl) and HbA_{1c} (% of Total Hb) levels in the study groups.

Groups	**FBS (mg/dl) Mean ±S.D**	**HbA_{1c} (%) Mean ±S.D**
DM with retinopathy	180.04 ± 68.51	7.86 ± 1.22
DM without retinopathy	176.24 ± 59.24	7.18 ± 1.05
Controls	90.20 ± 12.49	5.55 ± 0.68

As represented in the above Table 8, the mean FBS level in diabetic patients with retinopathy was 180.04 ± 68.51 mg/dl, in diabetic patients without retinopathy it was 176.24 ± 59.24 mg/dl and in the controls it was 90.20 ± 12.49mg/dl.

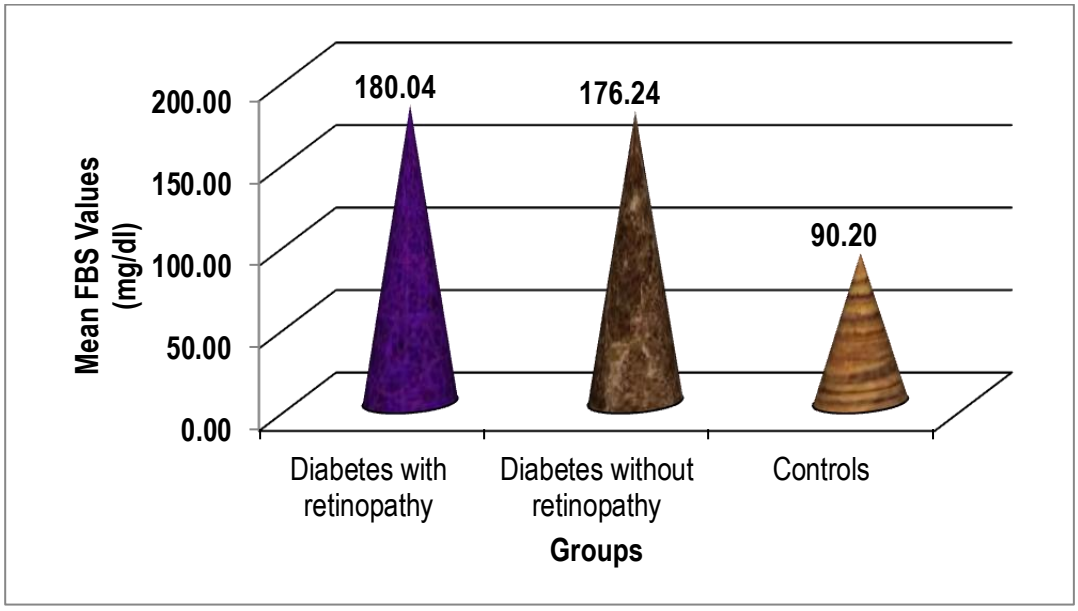
As represented in the above Table No 8, the mean HbA_{1c} levels in diabetic patients with retinopathy was 7.86 ± 1.22 %, in diabetic patients without retinopathy it was 7.18 ± 1.05 %, and in the controls it was 5.55 ± 0.68 %

Table No 9: Comparision of mean FBS and HbA_{1c} values between the study groups

FBS			
Between groups	**t- value**	**'p' value**	**Inference**
DM with DR & DM without DR	0.26	0.258	Not significant
DM with DR & controls	-6.5	< 0.001	Highly significant
DM without DR & controls	-7.1	< 0.001	Highly significant
HbA_{1c}			
Between groups	**t- value**	**'p' value**	**Inference**
DM with DR & DM without DR	-2.135	< 0.05	Significant
DM with DR & controls	-8.275	< 0.001	Highly significant
DM without DR & controls	-6.505	< 0.001	Highly significant

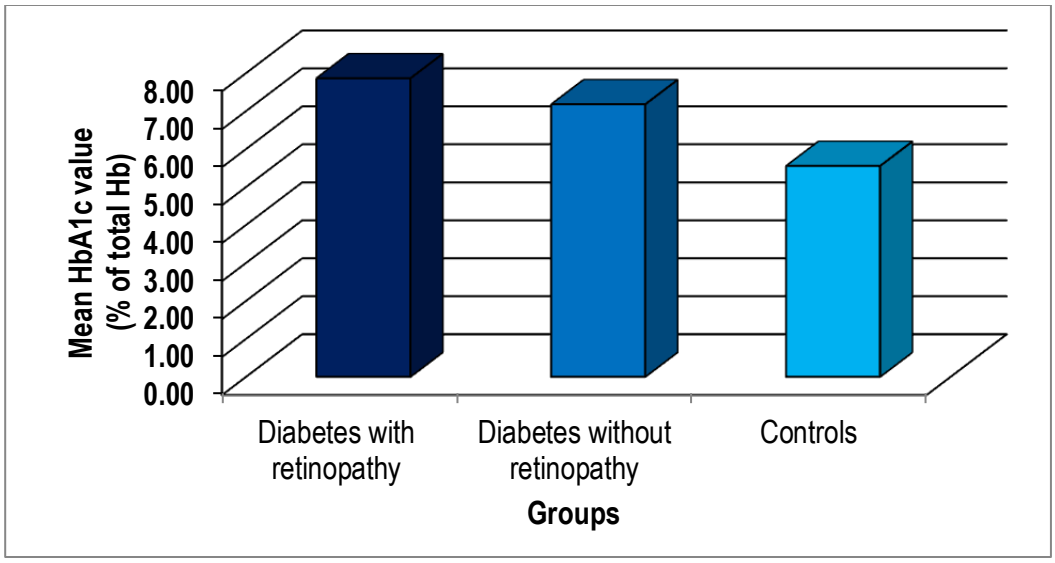
As represented in the above Table No 9, there was a significant increase in the mean FBS values in diabetic patients with retinopathy when compared to the diabetic patients without retinopathy ($p < 0.001$) and the controls ($p < 0.001$). Similarly, there was a significant increase in the mean FBS values in diabetic patients without retinopathy when compared to the control group ($p < 0.001$).

As represented in the above Table No 9, there was a statistically significant increase in the mean HbA_{1c} values in diabetic patients with retinopathy when compared to the diabetic patients without retinopathy ($p < 0.05$) and the control group ($p < 0.001$). Similarly, there was a significant increase in the mean HbA_{1c} values in diabetic patients without retinopathy when compared to the control group ($p < 0.001$).



Graph No 6: Mean FBS values (mg /dl) in the study groups

The above bar diagram shows that there is a significant increase in the mean FBS values in the diabetic patients with retinopathy when compared to the diabetic patients without retinopathy and the control group.



Graph No 7: Mean HbA_{1c} levels (%) in the study groups.

The above bar diagram shows that there is a significant increase in the mean HbA_{1c} values in diabetic patients with retinopathy when compared to the diabetic patients without retinopathy and the control group.

Table No 10: Glutathione (mg/gm of Hb) and Vitamin C(mg/dl) levels in study groups

Groups	Glutathione (mg/gm of Hb) Mean ±S.D	Vitamin C (mg/dl) Mean ±S.D
DM with retinopathy	6.14 ± 1.52	0.69 ± 0.25
DM without retinopathy	6.47 ± 1.69	0.86 ± 0.32
Control	13.14 ±2.48	1.26 ±0.25

As represented in the above Table No 10, the mean GSH levels, in diabetic patients with retinopathy was 6.14 ± 1.52 mg/gm of Hb, in diabetic patients without retinopathy it was 6.47 ± 1.69 mg/gm of Hb and in the control group it was 13.14 ± 2.48 mg/gm of Hb.

As represented in the above Table No 10, the mean Vitamin C level, in diabetic patients with retinopathy was 0.69 ± 0.25 mg/dl, in diabetic patients without retinopathy it was 0.86 ± 0.32 mg/dl and in the control group it was 1.26 ± 0.25 mg/dl.

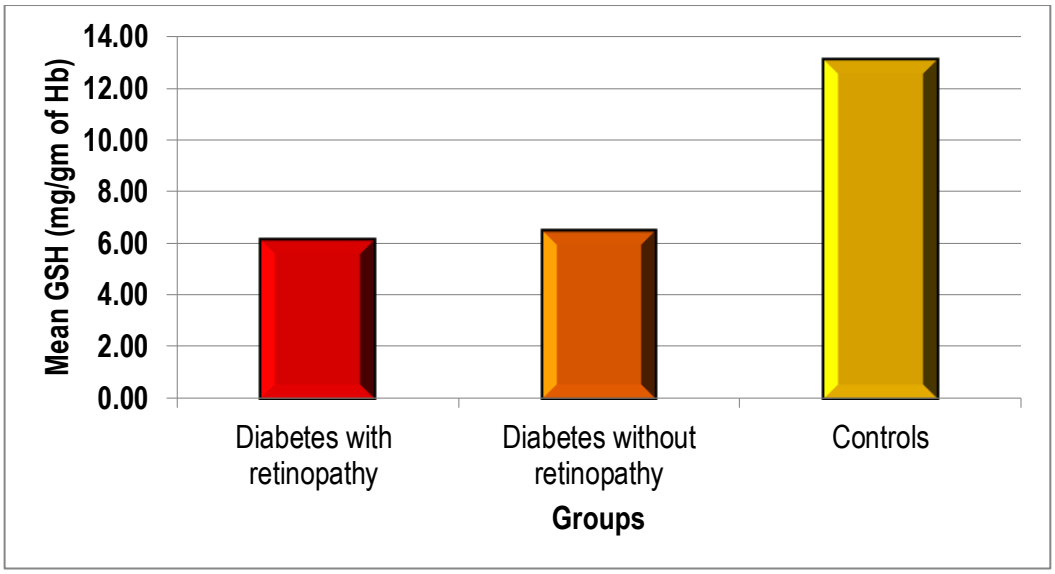
Table No 11: Comparision of mean Glutathione and vitamin C values between the study groups

Glutathione			
Between groups	**t-value**	**'p' value**	**Inference**
DM with DR & DM without DR	0.716	> 0.05	Not significant
DM with DR & controls	12.038	< 0.001	Highly significant
DM without DR & controls	11.128	< 0.001	Highly significant
Vitamin C			
Between groups	**t-value**	**'p' value**	**Inference**
DM with DR & DM without DR	2.182	< 0.05	Significant
DM with DR & controls	8.223	< 0.001	Highly significant
DM without DR & controls	4.927	< 0.001	Highly significant

As represented in the above Table No 11, there was no significant difference in the GSH levels in diabetic patients with retinopathy when compared to the diabetic patients without retinopathy ($p > 0.05$), but there was highly significant difference in the GSH levels in diabetic patients with retinopathy when compared to the controls ($p < 0.001$). Similarly, there was highly significant difference in the GSH levels in diabetic patients without retinopathy when compared to the controls ($p < 0.001$).

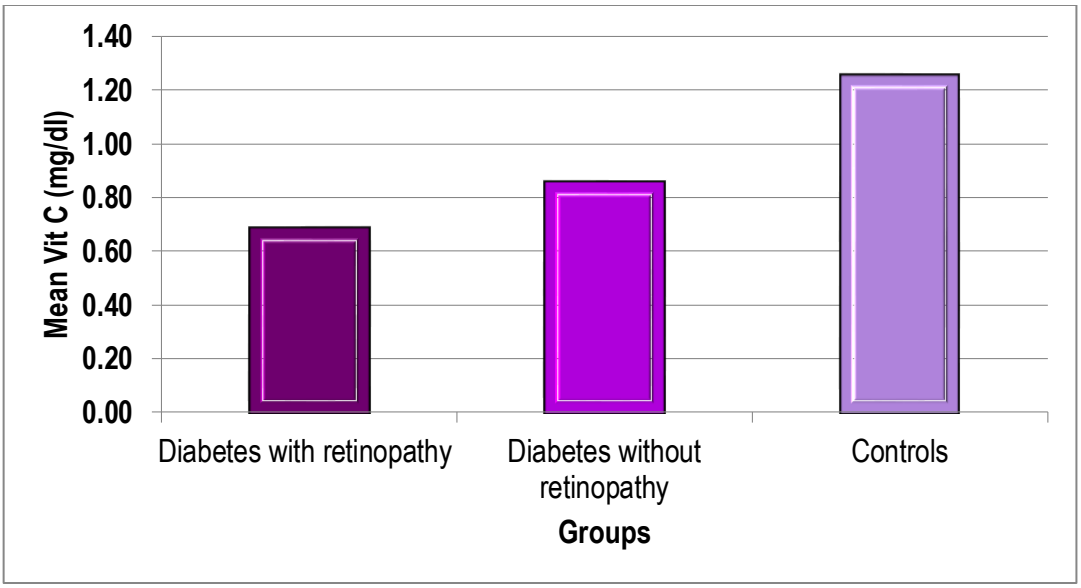
As represented in the above Table No 11, there was significant difference in the Vitamin C levels in diabetic patients with retinopathy when compared to the diabetic

patients without retinopathy ($p < 0.05$), but there was highly significant difference in the Vitamin C levels in diabetic patients with retinopathy when compared to the controls ($p < 0.001$). Similarly, there was highly significant difference in the Vitamin C levels in diabetic patients without retinopathy when compared to the controls ($p < 0.001$).



Graph No 8: GSH levels in the study groups

The above bar diagram shows that there is no significant difference in the mean GSH levels in diabetic patients with retinopathy when compared to the diabetic patients without retinopathy, but there was highly significant difference in the GSH levels in diabetic patients with retinopathy when compared to the controls. Similarly, there was highly significant difference in the GSH levels in diabetic patients without retinopathy when compared to the controls.



Graph No 9: Bar diagram showing Vitamin C levels in the study groups

The above bar diagram shows that there is significant difference in the mean Vitamin C levels in diabetic patients with retinopathy when compared to the diabetic patients without retinopathy, but there was highly significant difference in the Vitamin C levels in diabetic patients with retinopathy when compared to the controls. Similarly, there was highly significant difference in the Vitamin C levels in diabetic patients without retinopathy when compared to the controls.

Table No 12: Total cholesterol, Triglycerides, HDL and LDL in (mg/dl) in the study groups.

Groups	Total cholesterol (mg/dl) Mean ±S.D	Triglyceride (mg/dl) Mean ± S.D	HDL (mg/dl) Mean ±S.D	LDL (mg/dl) Mean ±S.D
DM with retinopathy	177.52 ± 44.82	201.16 ± 98.36	35.68 ± 3.97	106.84 ± 37.05
DM without retinopathy	197.88 ± 51.75	213.44 ±104.14	35.96 ± 4.22	118.80 ± 45.56
Control	176.64 ± 43.75	153.36 ± 76.27	37.24 ± 2.50	101.32 ± 35.66

As represented in the above Table No 12, the mean total cholesterol level in diabetic patients with retinopathy was 177.52 ± 44.82 mg/dl, in diabetic patients without retinopathy, it was 197.88 ± 51.75 mg/dl and in controls, it was 176.64 ± 43.75 mg/dl.

As represented in the above Table No 12 , the mean triglyceride level in diabetic patients with retinopathy was 201.16 ± 98.36 mg/dl, in diabetic patients without retinopathy, it was 213.44 ±104.14 mg/dl and in controls it was 153.36 ± 76.27 mg/dl.

As represented in above Table No 12, the mean HDL level in diabetic patients with retinopathy was 35.68 ± 3.97 mg/dl, in diabetic patients without retinopathy, it was 35.96 ± 4.22 mg/dl and in the control group it was 37.24 ± 2.50 mg/dl.

As represented in above Table No 12, the mean LDL levels, in diabetic patients with retinopathy was 106.84 ± 37.05 mg/dl, in diabetic patients without retinopathy it was 118.80 ± 45.56 mg/dl and in the control group it was 101.32 ± 35.66 mg/dl.

As represented in the below Table No 13, there was no statistically significant difference in the mean total cholesterol levels in diabetic patients with retinopathy, when compared to the diabetic patients without retinopathy ($p > 0.05$) and the control group ($p > 0.05$). Similarly, there was no statistically significant difference in the mean total cholesterol values in diabetic patients without retinopathy when compared to the control group ($p > 0.05$).

As represented in the below Table No 13, there was no statistically significant difference in the mean triglyceride levels in diabetic patients with retinopathy when compared to the diabetic patients without retinopathy ($p > 0.05$). But, there was statistically significant difference in the mean triglyceride values in diabetic patients

without retinopathy when compared to control group ($p < 0.05$). However there was a no statistically significant difference in the mean triglyceride levels in diabetic patients with retinopathy when compared to the control group ($p > 0.05$).

Table No 13: Comparison of lipid profile values in the study groups.

Total Cholesterol			
Between groups	**t-value**	**'p' value**	**Inference**
DM with DR & DM without DR	1.487	> 0.05	Not significant
DM with DR & controls	-0.070	> 0.05	Not significant
DM without DR & controls	-1.567	> 0.05	Not significant
Triglycerides			
Between groups	**t-value**	**'p' value**	**Inference**
DM with DR & DM without DR	0.428	> 0.05	Not significant
DM with DR & controls	-1.920	> 0.05	Not significant
DM without DR & controls	-2.327	< 0.05	Significant
HDL			
Between groups	**t-value**	**'p' value**	**Inference**
DM with DR & DM without DR	0.242	> 0.05	Not significant
DM with DR & controls	1.663	> 0.05	Not significant
DM without DR & controls	1.305	> 0.05	Not significant
LDL			
Between groups	**t-value**	**'p' value**	**Inference**
DM with DR & DM without DR	1.510	> 0.05	Not significant
DM with DR & controls	0.536	> 0.05	Not significant
DM without DR & controls	-1.018	> 0.05	Not significant

As represented in the above Table No 13, there was no statistically significant difference in the mean HDL levels in diabetic patients with retinopathy when compared to the diabetic patients without retinopathy ($p > 0.05$) and the control group ($p > 0.05$). Similarly there was no statistically significant difference in the mean HDL levels in diabetic patients without retinopathy when compared to control group ($p > 0.05$).

As represented in the above Table No 13, there was no significant difference in the LDL levels in diabetic patients with retinopathy when compared to the diabetic patients without retinopathy ($p > 0.05$) and the controls ($p > 0.05$). Similarly, there was no significant difference in the LDL levels in diabetic patients without retinopathy when compared to the controls ($p > 0.05$).

Table No 14: Correlation of Glutathione with other parameters

Parameter	Value	Diabetic Retinopathy	Diabetes mellitus	Controls
FBS	R	0.01	-0.14	-0.13
	P	0.95	0.49	0.55
Gly Hb	R	-0.07	-0.32	-0.34
	P	0.76	0.12	0.09
T. Cholesterol	R	-0.07	-0.44	-0.07
	P	0.74	0.03*	0.73
HDL Cholesterol	R	-0.01	0.37	0.21
	P	0.95	0.07	0.30
Triglycerides	R	-0.20	-0.19	-0.02
	P	0.34	0.37	0.91
LDL Cholesterol	R	0.02	-0.45	0.04
	P	0.91	0.02*	0.83
Vitamin C	R	0.13	0.20	-0.13
	P	0.55	0.34	0.54

The above Table No 14 shows that there was no significant correlation between GSH and other parameters in diabetic retinopathy and control groups. There was negative nt

correlation between GSH levels with Total cholesterol & LDL levels in diabetic mellitus group. This correlation was statistically significant.

Table No 15: Correlation of Vitamin C with other parameters

Parameter	Value	Diabetic Retinopathy	Diabetes mellitus	Controls
FBS	R	-0.17	0.25	0.41
	P	0.42	0.24	0.04*
Gly Hb	R	0.08	0.17	-0.04
	P	0.69	0.43	0.87
T. Cholesterol	R	0.07	0.08	0.17
	P	0.73	0.70	0.41
HDL Cholesterol	R	0.17	-0.11	0.11
	P	0.42	0.60	0.61
Triglycerides	R	-0.21	-0.17	0.00
	P	0.31	0.40	0.98
LDL Cholesterol	R	0.19	0.18	0.20
	P	0.37	0.38	0.33

The above table shows that there was no significant correlation between Vitamin C and other parameters in diabetic retinopathy and diabetic mellitus group. There was a significant correlation between Vitamin C with FBS levels in control groups.

DISCUSSION:

Diabetic Retinopathy is one of the microvascular complications of diabetes mellitus, which is one of the leading causes of acquired blindness.[50] It is due to microangiopathy affecting the retinal arterioles, capillaries and venules. Damage is caused by both microvascular leakage and microvascular occlusion. A series of risk factors have been related to the development and progression of retinopathy in diabetic patients

In our study, the number of cases in diabetic retinopathy group was found to be more between the age group of 61-70 yrs (56%).

In our study, it was observed that diabetic retinopathy was found to be higher in males when compared to females. A cohort study done in a clinic in Chennai on diabetic retinopathy appeared to be prevalent more in the males compared to females. A similar preponderance has been reported from the CURES Eye study[108] UKPDS study[109] and the Hyderabad study.[110] But in Joslin clinic patients, study reported that excess female preponderance over males.[111]

In our study, the mean duration of diabetes mellitus in diabetic patients with retinopathy was 13.2 ± 1.66 yrs and in diabetic patients without retinopathy it was 5.84 ±1.65 yrs. The duration of diabetes mellitus was significantly higher in diabetic patients with retinopathy when compared to diabetic patients without retinopathy.

Our findings are comparable with the study done by M Rema and R Pradeepa, who have proposed that duration of diabetes mellitus is probably the strongest predictor for the development of retinopathy. Studies have also shown that for every 5 year increase in the duration of diabetes mellitus, the risk of diabetic retinopathy increases by 1.89

times.[50] Studies done in Kuwait by Farhan K H and his associates, have also shown that duration of diabetes mellitus can increase the risk for the development of diabetic retinopathy.[112] The above findings show that duration of diabetes mellitus is one of the important risk factors in the development of diabetic retinopathy.

In our present study, there was a significant increase in the FBS levels and HbA_{1c} levels in diabetic patients with retinopathy and in diabetic patients without retinopathy when compared to the control group. The mean FBS level in diabetic patients with retinopathy was 180.04 ± 68.51 mg/dl, in diabetic patients without retinopathy it was 176.24 ± 59.24 mg/dl and in the controls it was 90.20 ± 12.49 mg/dl.

Our findings are comparable with the previous studies done around 2000 by Zelia Maria da silva correa and his co-workers, who have found that there was a significant association between FBS levels with the severity of diabetic retinopathy.[2] Ishrat kareem and associates, during the year 2002 have also observed increased FBS levels in diabetic patients with and without retinopathy when compared to the control group.[100]

The mean HbA_{1c} levels in patients with diabetic retinopathy was 7.86 ± 1.22%, in diabetic patients without diabetic retinopathy it was 7.18 ± 1.05 %, and in the controls it was 5.55 ± 0.68 %.

Our findings are comparable with the previous studies done by Ishrat Kareem and his coworkers, who have observed increased HbA_{1c} levels in diabetic patients with retinopathy and in diabetic patients without retinopathy.[100] Studies have also shown that in patients with type 2 diabetes mellitus, every 1%increase in HbA_{1c} would result in an

increase in the microvascular complications by 37%.[99] Study done in the year 2003 by K G Santos and his associates in south Brazil, have demonstrated a significant increase in HbA_{1c} levels in diabetic patients with retinopathy.[113]

The findings of the present study and the previous studies show that hyperglycemia, as indicated by the increase in the FBS and HbA_{1c} levels, is a potent predictor of progression to diabetic retinopathy. The possible mechanism is that hyperglycemia leads to glycation of virtually all proteins, resulting in the formation of advanced glycation end products. These advanced glycation end products induce cross linking of collagen and other extracellular matrix proteins in many tissues including arterial vessel walls.[114.]Hyperglycemia-induced vascular injury leads to increased glucose flux through the polyol pathway, resulting in cellular damage, thereby resulting in the various micro vascular and macro vascular complications.[115] HbA_{1c} is also shown to have a special affinity for oxygen thereby causes tissue anoxia and plays a role in causation of micro and macroangiopathy.[100] The interaction of advanced glycation end products and their receptors have been implicated as mediators of micro vascular permeability, ischemia & angiogenesis.[115]

The association between serum lipids and diabetic retinopathy has been investigated in many studies, which have shown conflicting results.

In the present study, the mean total cholesterol level in patients with diabetic retinopathy was 177.52 ± 44.82 mg/dl, in diabetic patients without retinopathy it was 197.88 ± 51.75 mg/dl and in controls it was 176.64 ± 43.75 mg/dl. There was no significant increase in diabetic group when compared to controls

The mean triglyceride level in patients with diabetic retinopathy was 201.16 ± 98.36mg/dl, in diabetic patients without retinopathy it was 213.44 ± 104.14 mg/dl and in controls it was 153.36 ± 76.27 mg/dl. There was significant increase in diabetic retinopathy and diabetic without retinopathy groups, when compared to controls.

Our findings does not match with the study done by Sinav and his coworkers, who have shown increase in the total cholesterol level with the severity of diabetic retinopathy.[102]Study done around 1980's by Dornan and his coworkers, does not agree with our findings, where there was no difference in triglyceride levels in diabetic patients with retinopathy when compared to the controls.[105]Study done in the year 1984 by Weber and his coworkers, on "Risk factors for the development of retinopathy in children and adolescents with type 1 diabetes mellitus" also found that there was no association between total cholesterol and diabetic retinopathy.[116]

In our present study, the mean GSH levels in diabetic patients with retinopathy were 6.14 ± 1.52 mg/dl, in diabetic patients without retinopathy it was 6.47 ± 1.69 mg/dl and in control group it was 13.14 ± 2.48mg/dl. There was a statistically significant decrease in the GSH levels in the diabetic groups compared to the control group.

In our present study, the mean Vitamin C levels, in diabetic patients with retinopathy was 0.69 ±0.25 mg/dl, in diabetic patients without retinopathy it was 0.86 ± 0.32 mg/dl and in the control group it was 1.26 ± 0.25 mg/dl. There was a significant decrease in the Vitamin C levels in diabetic retinopathy and diabetic without retinopathy groups, when compared to the control group.

We found that there was statistically negative significant correlation between GSH with Total cholesterol & LDL levels in diabetic mellitus group and there was statistically significant correlation between Vitamin C with FBS levels in control groups

Our findings agree with the study done in the year 2005 by Zuhal Yildirim and his coworkers, who have shown statistically significant decrease for GSH levels in diabetic retinopathy and diabetic without retinopathy groups when compared to control group.[117]

Another similar study published in Clin Chim Acta in year 2004, have shown statistically significant decrease of GSH levels in diabetic retinopathy and diabetic without retinopathy groups, when compared to control group and showed increased levels of lipid profile (TC,TG & LDL) [118]

Our findings agree with the study done in London in the year 1998, on "The role of oxidative stress in diabetic retinopthy" by Gurlar B and his associates.The study included 25 patients with diabetic retinopathy, 34 patients without retinopathy, and 26 healthy subjects who have shown statistically significant decrease of Vitamin C levels in diabetic retinopathy and diabetic without retinopathy groups, when compared to control group.[119]

Similar study done on "Plasma MDA and antioxidant vitamins in diabetic retinopathy" by S. Kumari and coworkers, who have shown statistically significant decrease of Vitamin C levels in diabetic retinopathy and diabetic without retinopathy groups, when compared to control group.[120]

These observations support the suggestion that chronic hyperglycaemia can influence the generation of free radicals, which may lead ultimately to increased lipid peroxidation and depletion of antioxidants, and thereby enhanced oxidative stress in subjects with type 2 diabetes mellitus.[121]

Our findings are comparable with previous studies done by Farhan K H and his associates, that the duration of diabetes, poor control of blood sugar and microalbuminuria increase the risk for the development of retinopathy.[112]

Zelia Maria da silva correa and coworkers found that the severity of diabetic retinopathy was influenced by duration of disease, glycemic control, total serum cholesterol and nephropathy.[2]

Studies have suggested that increased capillary permeability, microangiopathy and retinal ischemia are probably due to the combined effects of various risk factors.[122]

CONCLUSION:

Based on the results of the present study and data available from literature, it can be implicated that glycated haemoglobin (HbA_{1c}), oxidative stress parameters like erythrocyte glutathione (GSH) and Vitamin C are involved in the development of diabetic retinopathy.

Many studies, as we have already seen in the review of literature have shown that elevated HbA_{1c} levels are found to be the potent predictors of progression of diabetic retinopathy. Hyperglycemia leads to the formation of advanced glycation end products, which result in the various micro vascular complications.

GSH and Vitamin C levels were significantly altered in diabetes with complications and without complications when compared to normal, suggesting the role of uncontrolled hyperglycemia as a cause and consequence of oxidative stress.

The study emphasises that, prolonged uncontrolled diabetes, associated late complications like diabetic retinopathy, had a significantly increased measure of oxidative stress, when compared to the cases of uncomplicated diabetes. This suggests that the ongoing production and damage caused by reactive oxygen species had a strong relationship with diabetic status of an individual.

From the results of the present study, it can be concluded that estimation of HbA_{1c}, oxidative stress parameters like erythrocyte glutathione and vitamin c can be considered as a good index predicting the onset and progression of diabetic retinopathy.

Strict control of blood glucose as determined by fasting blood glucose and glycosylated haemoglobin is necessary to prevent the onset of complications associated with diabetes mellitus. However, with poor glycemic control, oxidative stress in diabetes can cause profound damage to the vital organs in the body. Hence determination of reduced glutathione in erythrocytes and vitamin c levels can contribute to know the extent of oxidative stress in diabetes and help in effective control and prevention of the onset and progression of complications like diabetes retinopathy.

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SUMMARY:

Diabetic retinopathy is one of the leading causes of blindness in the world that increases the chance of losing the sight about 25 times higher compared to normal individuals. Various precipitating factors such as duration of disease, Sex preponderance, glycemic control, dyslipidemia and oxidative stress parameters (erythrocyte glutathione, vitamin c) have been implicated in the development and progression of diabetic retinopathy.

Therefore the present study was done at R.L.Jalappa Hospital & Research Center to study the role of these precipitating factors in diabetic patients with retinopathy and in diabetic patients without retinopathy and comparing it with healthy controls.

- 25 diabetic patients with retinopathy, 25 diabetic patients without retinopathy, 25 controls were selected for the study
- There was an increase in the FBS, HbA_{1c} and Triglyceride levels in diabetic patients with retinopathy, when compared to the diabetic patients without retinopathy and the controls. The increase was statistically significant.
- There was a decrease in the Glutathione, Vitamin C levels in diabetic patients with retinopathy when compared to the diabetic patients without retinopathy and the controls and it was statistically significant.
- There was increase in FBS, HbA_{1c}, and Triglyceride levels in the diabetic patients without retinopathy when compared to the controls and was statistically significant.
- There was significant correlation between GSH with total cholesterol and LDL in diabetic mellitus when compared to the diabetic patients without retinopathy

and the controls. There was significant correlation between Vitamin C with FBS in controls when compared to the diabetic patients without retinopathy and diabetic patients with retinopathy.

Early diagnosis and prompt treatment of the complications of diabetes mellitus, such as retinopathy, can improve the quality of life and increase life expectancy

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Annexure No 1:

PROFORMA

Case No	:	IP No	:
Name	:	HP No	:
Age	:	Ward	:
Sex	:	Hospital	:
Occupation	:	Date	:
Address	:		

PRESENTING COMPLAINTS:

HISTORY OF PRESENTING COMPLAINTS:

PAST HISTORY

Hypertension	: Yes / No	Duration
Tuberculosis:	: Yes / No	Duration
Diabetes:	: Yes / No	Duration
On medications	: Yes / No	Duration

DIABETIC HISTORY:

Duration of diabetes:

Associated complications:

Drugs used for diabetes mellitus- OHA / Insulin / Insulin + OHA

PERSONAL HISTORY:

Economic status:

Smoking : Yes / No Duration

Alcohol : Yes / No Duration

Diet : Veg/ Mixed

GENERAL PHYSICAL EXAMINATION:

Built: Nourishment :

Pallor / clubbing / lymphadenopathy / icterus / cyanosis / oedema.

Pulse - ----------/min BP - ---------- mmHg

SYSTEMIC EXAMINATION

Respiratory system :

Cardiovascular system :

Gastrointestinal system :

Central nervous system :

DIAGNOSIS:

INVESTIGATIONS:

Blood

Hb% **:** ----------gms/dl

Blood sugar (FBS) : ----------mg/dl

Glycated Hb : ----------%

Lipid Profile :

Total cholesterol : ----------mg/dl

Triglycerides : ----------mg/dl

High density lipoprotein : ----------mg/dl

Low density lipoprotein :----------mg/dl

Very Low density lipoprotein : ----------mg/dl

Other investigations

Oxidative stress parameters :

Reduced Glutathione :

Vitamin C :

Direct opthalmoscopy

To detect and grade Retinopathy

Non-Proliferative Diabetic Retinopathy :

Proliferative Diabetic Retinopathy :

Annexure 2: Master Chart: 01 Controls

Sl. No	Gender	Age in yrs	Hospital No	FBS (mg%)	Glycosylated Hb (%)	GSH (mg%)	Vit C (µg%)	TC(mg%)	TG (mg%)	HDL (mg%)	LDL (mg%)	VLDL (mg%)
1	F	34	586269	72	4.8	14.46	1.2	142	107	39	82	21.4
2	F	29	457150	92	4.6	16.2	1.2	193	160	39	122	32
3	M	38	627312	116	5	11.43	1.6	265	231	33	185	46.2
4	F	39	503245	104	4.6	12.5	1.2	197	182	39	121	36.4
5	M	51	630607	88	6.39	11.43	1.2	208	200	39	129	40
6	M	56	630940	87	6.05	6.57	1.6	160	161	39	88	32.2
7	M	65	630856	94	5	12.96	1.2	140	96	37	83	19.2
8	M	53	626672	83	6	12.5	1.2	136	168	39	63	33.6
9	M	69	627364	83	6.22	13.5	1.6	141	84	32	92	16.8
10	M	52	509051	106	6.05	13.46	1.2	140	60	38	90	12
11	M	61	627386	94	6.14	11.21	1.2	255	242	35	171	48.4
12	F	50	642812	82	4.6	16.66	1.6	150	56	40	98	11.2
13	M	65	642816	88	5	14.58	1.2	165	87	39	108	17.4
14	M	51	631311	92	6.97	8.15	1.2	209	102	36	102	20.4
15	F	62	631313	108	5.46	10.81	1.6	171	84	35	119	16.8
16	M	66	631317	60	4.79	13.79	1.2	103	74	38	50	14.8
17	F	56	631316	79	5.55	12.86	0.8	209	213	35	131	42.6
18	F	60	631308	82	6	14.34	1.2	231	232	40	144	46.4
19	M	45	631615	73	5.3	13.15	0.8	111	143	37	45	28.6
20	M	42	605152	100	4.79	16.07	1.2	179	359	36	84	71.8
21	M	62	632884	101	6.22	11.66	1.6	149	277	38	55	55.4
22	F	53	632885	98	6.05	16.3	1.2	172	118	39	109	23.6
23	M	40	633011	85	6.05	13.88	0.8	150	112	31	96	22.4
24	M	40	634482	90	6	13.15	1.2	186	92	39	128	18.4
25	F	50	628562	98	5.2	17	1.6	254	194	39	176	38.8

Master Chart: 02 Diabetes Mellitus without complications

Sl. No	Gender	Age in yrs	Hospital No	FBS (mg%)	Glycosylated Hb (%)	GSH (mg%)	Vit C (µg%)	TC (mg%)	TG (mg%)	HDL (mg%)	LDL (mg%)	VLDL (mg%)	Duration of DM (yrs)
1	M	68	555037	162	6.81	6.12	0.8	182	133	36	119	26.6	8
2	M	50	624608	179	6.7	4.34	0.4	231	192	36	156	38.4	6
3	M	43	625218	108	4.5	6.84	0.8	182	130	39	117	26	5
4	M	50	624838	164	5.7	7.04	0.8	261	400	47	134	80	6
5	M	70	624842	126	6.9	6.19	0.4	101	115	37	41	23	4
6	M	57	625793	186	9.9	2.62	0.8	266	400	32	154	80	6
7	M	56	621292	114	6.8	9.8	0.8	140	69	38	88	13.8	5
8	M	65	625565	206	8.2	5.55	0.8	261	268	28	179	53.6	8
9	M	50	626483	118	7	6.88	0.8	100	104	37	42	20.8	5
10	F	60	624581	183	8	7.93	1.2	182	130	39	117	26	6
11	F	65	624102	276	8	7	0.8	186	170	33	119	34	5
12	M	65	600709	317	7.2	8.26	1.6	156	270	34	68	54	2
13	M	68	628677	119	7	6.57	1.6	228	132	33	168	26.4	5
14	M	72	619915	220	7.73	6.41	1.2	253	154	37	185	30.8	3
15	M	35	630612	282	7.89	5.06	0.8	212	308	25	125	61.6	7
16	M	86	624013	158	7	4.39	1.2	140	89	37	85	17.8	8
17	M	61	630482	99	6.05	5.99	0.8	200	335	34	95	67	4
18	M	50	627707	188	7.73	6.32	1.2	276	158	39	205	31.6	6
19	M	55	626305	127	8	8.75	0.8	174	275	38	81	55	8
20	F	55	617232	258	7.06	5.2	0.8	283	282	32	194	56.4	5
21	F	53	631194	189	6.81	5.95	0.4	203	167	39	130	33.4	8
22	M	48	631147	190	6.88	10	0.8	138	200	37	61	40	6
23	M	65	631307	189	8.65	4.87	0.8	179	121	35	119	24.2	5
24	F	48	632928	105	6.55	7.69	0.8	201	378	38	87	75.6	8
25	M	60	632926	143	6.39	5.87	0.4	212	356	39	101	71.2	7

Master Chart: 03 Diabetes Mellitus with complications

Sl. No	Gender	Age in yrs	Hospital No	FBS (mg%)	Glycosylated Hb (%)	GSH (mg%)	Vit C (µg%)	TC (mg%)	TG (mg%)	HDL (mg%)	LDL (mg%)	VLDL (mg%)	Duration of DM (yrs)
1	F	65	622556	89	8	9.43	0.8	219	159	40	147	31.8	15
2	F	40	620609	138	6.1	4.46	0.4	162	150	39	93	30	12
3	F	65	587016	123	8.23	6.06	0.8	258	267	33	171	53.4	14
4	F	66	625811	109	9.46	4.38	1.2	205	176	39	130	35.2	13
5	M	40	627694	234	8.23	5.13	0.4	167	381	25	65	76.2	10
6	M	70	598159	124	7.39	5.83	0.4	187	300	41	86	60	16
7	M	65	627693	102	8.31	5.16	0.8	185	187	42	105	37.4	12
8	M	60	603039	163	8.23	6.35	0.8	229	400	31	118	80	11
9	M	64	605907	195	8.73	7.5	0.4	120	185	33	50	37	14
10	M	75	627595	111	8.73	5.47	0.8	227	389	35	114	77.8	15
11	M	62	596170	199	5.8	5	0.8	129	165	38	58	33	12
12	M	60	627793	131	8.48	4.62	0.4	110	100	30	60	20	16
13	F	56	610980	90	8.4	5.85	0.8	210	184	36	137	36.8	14
14	M	55	629137	156	5.8	10	0.8	95	103	32	42	20.6	11
15	M	61	61278	223	6.64	6.25	1.2	120	60	33	75	12	12
16	M	69	630165	119	9	4.03	0.8	194	202	35	118	40.4	15
17	F	65	630548	265	7.89	6.25	0.8	140	188	36	66	37.6	13
18	M	65	553219	327	7.89	5.76	0.8	130	61	39	78	12.2	14
19	M	45	630229	281	4.46	5.03	0.4	217	374	40	102	74.8	12
20	M	55	628132	277	9.65	6.52	0.4	184	104	32	131	20.8	14
21	M	55	628095	198	8	5.47	0.4	132	152	35	66	30.4	11
22	F	70	565177	278	8.2	6.25	0.8	186	156	35	119	31.2	14
23	M	70	622527	184	8.6	6.25	0.4	240	202	37	163	40.4	13
24	F	45	614949	217	8.4	7.9	0.8	196	204	37	118	40.8	15
25	M	45	629137	168	8	8.57	0.8	196	180	39	121	36	12