RED BLOOD CELL MORPHOLOGY AS A MARKER OF OXIDATIVE STRESS IN EARLY TYPE 2 DIABETES PATIENTS AND EFFICACY OF ANTIOXIDANTS AS AN ADD ON THERAPY TO STANDARD TREATMENT - A RANDOMIZED, OPEN LABEL, COMPARATIVE PILOT STUDY

Dissertation submitted to

THE TAMILNADU

DR. M.G.R. MEDICAL UNIVERSITY

In partial fulfillment for the award of the degree of

DOCTOR OF MEDICINE

IN

PHARMACOLOGY

INSTITUTE OF PHARMACOLOGY
MADRAS MEDICAL COLLEGE
CHENNAI - 600 003

OCTOBER 2016

CERTIFICATE

This is to certify that the dissertation entitled, **"RED BLOOD CELL MORPHOLOGY AS A MARKER OF OXIDATIVE STRESS IN EARLY TYPE 2 DIABETES PATIENTS AND EFFICACY OF ANTIOXIDANTS AS AN ADD ON THERAPY TO STANDARD TREATMENT - A RANDOMIZED, OPEN LABEL, COMPARATIVE PILOT STUDY"** submitted by DR. ROHINI ANN MATHEW, in partial fulfilment for the award of the degree of Doctor of Medicine in Pharmacology by The Tamil Nadu Dr.M.G.R.Medical University, Chennai is a bonafide record of the work done by her in the Institute of Pharmacology, Madras Medical College during the academic year 2013-16.

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CERTIFICATE OF THE GUIDE

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DECLARATION

I, Dr. ROHINI ANN MATHEW solemnly declare that the dissertation titled "**RED BLOOD CELL MORPHOLOGY AS A MARKER OF OXIDATIVE STRESS IN EARLY TYPE 2 DIABETES PATIENTS AND EFFICACY OF ANTIOXIDANTS AS AN ADD ON THERAPY TO STANDARD TREATMENT - A RANDOMIZED, OPEN LABEL, COMPARATIVE PILOT STUDY"** has been prepared by me and submitted to TN Dr.MGR Medical University, Chennai in partial fulfillment of the rules and regulations for the M.D degree examination in Pharmacology.

Date: DR. ROHINI ANN MATHEW

Place:

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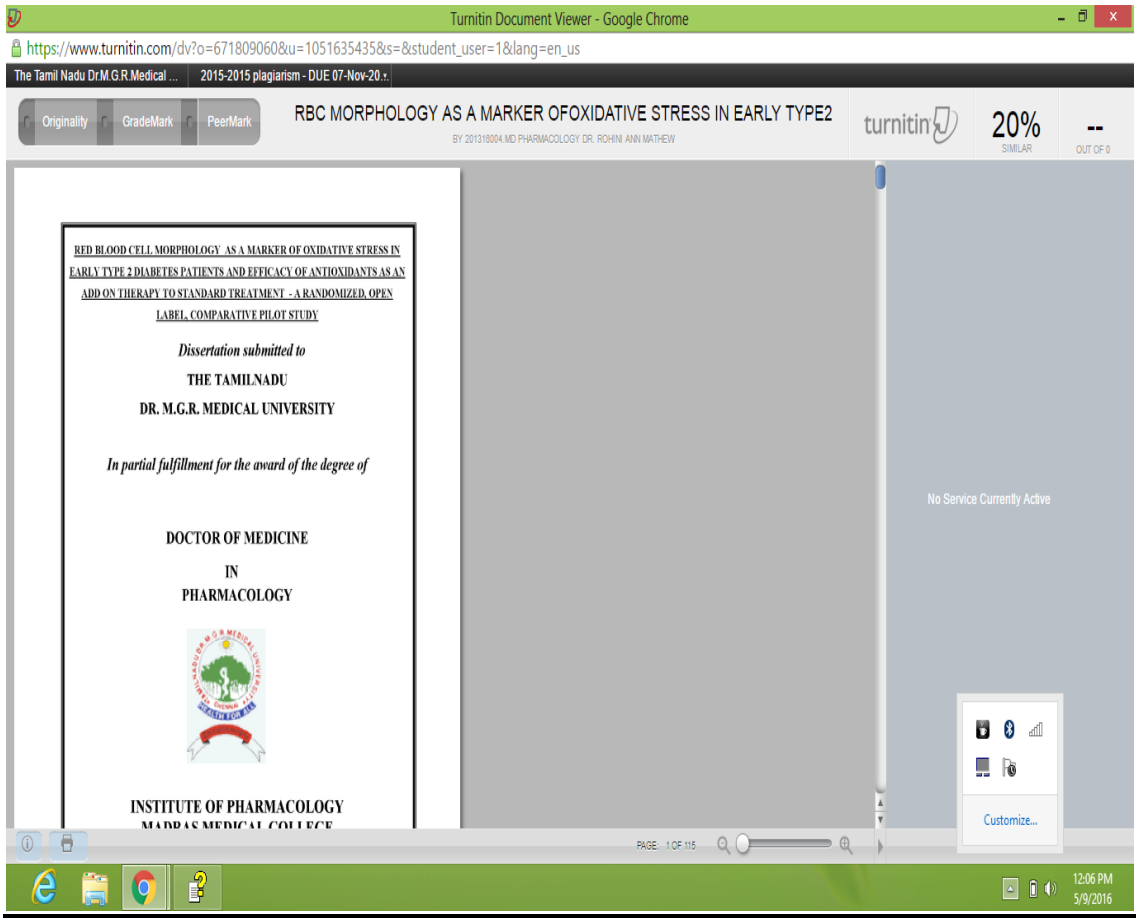

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ABBREVIATIONS

DM - Diabetes Mellitus

WHO - World Health Organization

ROS - Reactive oxygen species

RBC - Red blood cell

PPARα - peroxisome proliferator activated receptor alpha

FFA - free fatty acid

BMI - Body mass index

ECG - Electro cardiography

GLP1 - Glucagon like peptide 1

DPP 4 - Dipeptidyl peptidase 4

SGLT 2 - Sodium glucose co-transporter 2

DNA - Deoxy ribonucleic acid

GIP - Gastric inhibiting polypeptide.

ATP - Adenosine triphosphate

AMPK - AMP dependant protein kinase

DKA - Diabetic ketoacidosis

PEP - Phospho enol pyruvate

TAG -Tri acyl glycerol

TNF α -Tumor necrosis factor alpha

IL-6 - Interleukin 6

IL-1 - Interleukin 1

iNOS - inducible Nitric oxide synthase

IRS - insulin receptor substrate

MAP - Mitogen activated protein kinase

GLUT - Glucose transporter

Lep-Rb - Leptin receptor b

RNS - Reactive nitrogen species

NADPH - Nicotinamide adenine dinucleotide phosphate

AGE - Advanced glycation end products

PKC - Protein kinase C

DAG - Di acyl glycerol

NF-kB - Nuclear factor kappa B

NO - Nitric oxide

CFU –E - Colony forming unit – erythrocyte

HMP - Hexose monophosphate

G6PD - Glucose 6-phosphate dehydrogenase

GSH - Glutathione

LDL - Low density lipoprotein

IgG - Immunoglobulin G

IgM - Immunoglobulin M

CYP - Cytochrome P

PUFA - Poly unsaturated fatty acids

ANOVA - Analysis of variance

CONTENTS

INTRODUCTION

INTRODUCTION

Diabetes mellitus is a heterogeneous group of metabolic disorders characterized by chronic hyperglycemia resulting from defects in insulin secretion, insulin action or both [1].

It is classified into 2 major types: insulin dependent (Type 1) and non-insulin dependent (Type 2) [2].

Type 1 diabetes mellitus is characterized by a specific destruction of the pancreatic beta cells mostly associated with immune mediated damage [3].

Type 2 diabetes mellitus is characterized by a gradual change in glucose homeostasis due to insulin resistance and/or reduced insulin secretion [4].

Type 2 diabetes mellitus is now taking its place as one of the main threats to human health in the 21st century[5]. The World Health Organization (WHO) has estimated that by 2025 there will be about 300 million people living with diabetes worldwide, with India alone contributing a massive 57.2 million [6].

Though the metabolic derangements in Type 1 diabetes can be easily explained due to lack of insulin, for Type 2 diabetes it is multi-factorial influenced by genetic and environmental factors.

Oxidative stress plays an important role in the pathogenesis of Type 2 diabetes and its complications [7]. In diabetic patients there is a significant decrease in the

activity of enzymatic antioxidant defense system like Superoxide dismutase, glutathione reductase, glutathione peroxidase and catalase. [8]

Reactive oxygen species (ROS) generated from chronic hyperglycemia in these patients disrupts the critical balance between oxidants and antioxidants [9] and is implicated in the development of long-term microvascular and macrovascular complications associated with high morbidity and mortality in these patients [10].

Free radicals are highly reactive molecular species with an unpaired electron that can cause damage to nucleic acids, proteins and lipids in the cell membrane and plasma lipoproteins. Tissue damage caused by these free radicals is often termed as "**Oxidative damage**"[11].

Red blood cells (RBCs) are highly susceptible to oxidative damage as they are the first cells to be exposed to oxidative stress. Presence of high cellular concentration of oxygen and hemoglobin, lack of nucleus and mitochondria, inability to synthesize enzymes and protein make RBCs vulnerable to oxidative stress [12].

Therefore, structural damage like crenated edges and Heinz bodies in RBCs caused by ROS can be used as a marker of oxidative stress in Diabetes mellitus.

The level of antioxidants is reduced in diabetic patients and supplementation of non enzymatic antioxidants like Vitamin E and C can reduce this oxidative damage.

Therefore in this study RBC morphology is used as a marker of oxidative stress in Type 2 Diabetes and the effect of antioxidants like Vitamin E and C is studied in reversing this oxidative damage.

REVIEW OF

LITERATURE

LITERATURE REVIEW

DIABETES MELLITUS

DEFINIITON

Diabetes mellitus is characterized by chronic hyperglycemia with disturbances of carbohydrate, fat, and protein metabolism resulting from defects in insulin secretion, insulin action, or both. [13]

ETIOLOGICAL CLASSIFICATION [1]

1. Type 1 (beta cell destruction leading to absolute insulin deficiency)
    - Autoimmune
    - Idiopathic

2. Type 2 (hyperglycemia, insulin resistance and relative insulin deficiency)

3. Other specific types: - Genetic defects of β-cell function/insulin action
    - Endocrinopathies
    - Drug- or- chemical-induced
    - Infections

4. Gestational diabetes.

EPIDEMIOLOGY [6,7]

The worldwide prevalence of Diabetes Mellitus has risen dramatically over the past two decades. The WHO has estimated a rise from an about 150 million cases in 2000 to 300 million by 2025. Although the prevalence of both type 1 and type 2 DM is increasing worldwide, the prevalence of type 2 DM is rising much more rapidly due to increasing obesity and reduced physical activity. This is true in most countries, and 6 of the top 10 countries with the highest rates are in Asia with India topping the list.

Diabetes mellitus increases with aging. The prevalence is similar in men and women throughout most age ranges (10.5% and 8.8% in individuals >20 years) but is slightly greater in men >60 years. Worldwide estimates project that in 2030 the greatest number of individuals with diabetes will be in the range of 45–64 years.

TYPE 1 DIABETES MELLITUS: [1,6]

Type 1 diabetes occurs primarily due to β–cell destruction which produces a state in which insulin is required for survival.

It is sub-classified as:

- Type 1A: immune-mediated
- Type 1B: idiopathic

TYPE 2 DIABETES MELLITUS:

Type 2 diabetes is the commonest form of diabetes worldwide. These patients usually have insulin resistance with a state of relative, rather than absolute, insulin deficiency. The specific etiology of this form of diabetes is not known. Although these patients do not need insulin therapy to survive, ultimately many require it for control of blood glucose. Type 2 diabetes is associated with progressive β -cell failure with increasing duration of the disease.[1]

PATHOGENESIS[6,7]

Type 2 diabetes appears to develop due to a complex interplay of acquired (diet- or obesity- or stress related) and genetically programmed insulin resistance wherein the β-cells of the pancreas fail to produce the extra insulin needed to maintain normal blood glucose levels.

The relationship between plasma insulin and glucose in these patients can be depicted as occurring in 3 phases:

- **Phase I**: glucose levels are normal because of an underlying hyperinsulinemia which occurs to compensate for the insulin resistance at the level of the muscle, liver and other tissues.

- **Phase II**: insulin resistance and compensatory hyperinsulinemia progress but the pancreatic β-cells are no longer able to sustain the hyperinsulinemic state. Impaired glucose tolerance, characterized by elevations in postprandial glucose, then develops.
- **Phase III**: A further decline in insulin secretion and an increase in hepatic glucose production lead to overt diabetes with fasting hyperglycemia. Ultimately, β-cell failure occurs.

RISK FACTORS:

1. OBESITY:

In obesity there is an increase in pro-inflammatory molecules called "adipokines" that induce systemic insulin resistance by direct effects on insulin signaling, down-regulation of genes needed for normal insulin action and negative regulation of PPARα.[14] Increase in visceral fat mass leads to unrestrained lipolysis. This elevates circulating FFA levels resulting in an increase in the delivery of FFA to the liver by the portal vein. This leads to hepatic insulin resistance and decreased insulin clearance, with secondary effects of peripheral insulin resistance.[15]

2. **PHYSICAL ACTIVITY:**

Physical activity has been found to be inversely related to future risk of diabetes in most populations. Increased physical activity reduces the risk of obesity. This is related to

acute and long-term improvements in insulin sensitivity and reduction in insulin concentrations.[16]

3. ENVIRONMENTAL INFLUENCES:[17,18]

Sedentary life style, Dietary habits (consumption of high fat, low fibre diet) have been shown to increase the risk of diabetes in most populations.

4.GENETIC FACTORS:[1]

A strong genetic basis is suggested by the high prevalence of insulin resistance in certain populations. These include the Nauru Islanders of the Pacific, the Pima Indians in Arizona, and the urban Wanigela people in Papua New Guinea. Also, there is a nearly 100% concordance in diagnosis of type 2 diabetes between monozygotic twins but only a 20% concordance between dizygotic twins.

5.STRESS AND HORMONAL IMBALANCES[1]

During exercise and under conditions of stress, catecholamines are released from the adrenal medulla or by sympathetic nerve terminals in the pancreas. They activate α_2-adrenoceptors in β-cells of pancreas and reduce insulin secretion and increase glucagon release. Increase in counter-regulatory hormones (glucagon, epinephrine etc) creates a

hormonal imbalance resulting in elevated blood glucose. This partly accounts for the worsening glycemic control seen in individuals with diabetes who are under severe stress.

CLINICAL PRESENTATION [19,20]

The clinical onset may be over several months to years, particularly in older patients. Polyuria (increased frequency of micturation), Polydipsia (increased thirst), Polyphagia (increased appetite) and weight loss are the most common symptoms. These may be accompanied by other complaints such as lack of energy, visual blurring (owing to glucose-induced changes in refraction), or pruritis vulvae or balanitis due to Candida infection.

Patients with Type 2 diabetes may be asymptomatic and diagnosed only incidentally on routine examination.

PHYSICAL EXAMINATION [19,20]

A complete physical examination in diabetics should include :

- Weight or BMI,
- Blood pressure
- Retinal examination,
- Peripheral pulses.

- Lower extremities for peripheral neuropathy, calluses, superficial fungal infections, nail changes, ankle reflexes, and foot deformities (such as hammer or claw toes and Charcot foot) to identify sites of potential skin ulceration.
- Teeth and gums for periodontal disease
- Acanthosis nigricans for insulin resistance.

COMPLICATIONS [19]

ACUTE COMPLICATIONS

- Diabetic ketoacidosis
- Non ketotic hyperglycemic hyperosmolar coma

CHRONIC COMPLICATIONS

- Micro vascular
  - Retinopathy - Neuropathy - Nephropathy
- Macro vascular
  - Coronary artery disease - Cerebrovascular disease
  - Peripheral arterial disease
- Others
  - Gastro intestinal (gastroparesis/diarrhoea)
  - Genitor urinary (uropathy /sexual dysfunction)
  - Dermatological infections
  - Cataract/Glaucoma

- Periodontal disease

INVESTIGATIONS [19,20]:

- Blood sugar level: Fasting and Post Prandial.
- Urine sugar level (not reliable)

TO ASSESS THE DEGREE OF GLYCEMIC CONTROL

- HbA1C

FOR DIABETES RELATED CONDITIONS:

- Full blood count
- Urine for protein
- Serum Urea and Creatinine (renal function tests)
- Serum electrolytes
- Serum lipid profile
- Liver biochemistry
- Fundus examination
- ECG

DIAGNOSTIC CRITERIA[21]

- Symptoms of diabetes plus random blood glucose concentration $\geq$200 mg/dL (11.1mmol/L) or

- Fasting plasma glucose ≥126 mg/dL (7.0 mmol/L) or
- Two-hour plasma glucose ≥200 mg/dL (11.1mmol/L) during an oral glucose tolerance test or

- HbA_{1c} ≥ 6.5%

MANAGEMENT [22,23]

1. Glycemic control
    - Specific medications
2. Treatment of associated conditions
    - Dyslipidemia
    - Hypertension
    - Obesity etc.
3. Screening and management of complications
    - Retinopathy
    - Nephropathy
    - Neuropathy
    - Cardiovascular diseases

GOALS OF THERAPY IN DIABETES [21]

INDEX	VALUE
HbA1c	<7.0%
Pre prandial capillary plasma glucose	70-130 mg/dl
Post prandial capillary plasma glucose	<180 mg/dl
Blood pressure	<130/80 mmHg

NON PHARMACOLOGICAL MANAGEMENT[1]:

1. Diet management
    - Adequate amount of carbohydrates (40%), rich in dietary fibre, low in saturated fat.
2. Exercise
    - Aerobic activity 5-7 days/week, at a level that can be sustained for at least 30 minutes, with the maximum heart rate not any higher than 60% to 70% above the resting.
3. Stress reduction
    - Yoga, meditation ,relaxation therapy
    - Breathing exercises.

MEDICAL MANAGEMENT[21,22,23]

ORAL HYPOGLYCEMIC AGENTS (OHA)

A. Drugs acting by release of insulin

1. ***Sulfonylureas***
   - First generation : Chlorpropamide, Tolbutamide
   - Second generation: Glibenclamide, Glipizide, Gliclazide, Glimepride
2. ***Meglitinide analogues***: Repaglinide, Nateglinide
3. ***Glucagon like peptide (GLP 1) receptor agonists*** (injectables) : Exenatide, Liraglutide
4. ***Dipeptidyl peptidase -4 (DPP 4) inhibitors***: Sitagliptin, Vildagliptin, Saxagliptin, Alogliptin, Linagliptin

B. Reducing insulin resistance

1. ***Biguanides***: Metformin
2. ***Thiazolidinediones***: Pioglitazone

C. Miscellaneous

1. ***α glucosidase inhibitors:*** acarbose, miglitol, voglibose
2. ***Amylin analogues :*** Pramlintide
3. ***Dopamine (D2) receptor agonists:*** Bromocriptine
4. ***Sodium – Glucose cotransport -2 (SGLT -2) inhibitor:*** Dapaglifozin

SULFONYLUREAS:

Blocks ATP sensitive potassium channels in pancreatic β-cells

Adverse Effects: Hypoglycemia, weight gain.

MEGLITINIDES/D-PHENYLALANINE ANALOGUES:

- K_{ATP} channel blockers

Adverse Effects: Dizziness, dyspepsia, flu like symptoms

GLUCAGON-LIKE PEPTIDE 1 (GLP-1) RECEPTOR AGONISTS

GLP-1 is an important incretin released from the gut in response to ingested glucose

- Induces insulin release from β-cells
- Inhibits glucagon release from α-cells
- Slows gastric emptying and suppresses appetite

Adverse Effects: Nausea, vomiting, diarrhoea

DIPEPTIDYL PEPTIDASE-4 (DPP) INHIBITORS:

DPP -4 causes rapid degradation of endogenous GLP-1 which is an insulin secretagogue.

- Competitive and reversible inhibitor of DPP 4

- Decreases metabolism of GLP-1, potentiates its action

Adverse Effects: Nausea, loose stools, allergic reactions

BIGUANIDES (METFORMIN)

AMPK (AMP dependent protein kinase) activator

- Inhibits hepatic glucose production and release
- Enhances insulin mediated glucose uptake in peripheral tissues
- Promotes peripheral glucose utilization

Uses:

- First line drug for all Type 2 diabetics
- Good anti hyperglycemic action
- Promotes weight loss
- Potential to prevent diabetic complications

Adverse Effects: GI intolerance, metallic taste, megaloblastic anemia

THIAZOLIDINEDIONES:

- Agonist of PPAR $_{\Upsilon}$ (peroxisome proliferator activated receptor)
- Enhances transcription of insulin sensitive genes and increases glucose uptake in fat and muscle.

Adverse Effects: Weight gain, precipitation of Congestive heart failure, hepatotoxicity

α-GLUCOSIDASE INHIBITORS:

- Prevents the conversion of complex carbohydrates to simple carbohydrate by α-glucosidase inhibition and reduces its absorption

Adverse Effects: GI disturbances, elevation of liver enzymes

AMYLIN ANALOGUES:

- Delays gastric emptying
- Suppresses glucagon secretion

Adverse Effects: Nausea, hypoglycaemia

BROMOCRIPTINE (D_2 AGONIST):

- Dopaminergic control over circadian rhythm of anti-insulinic hormones to reduce insulin resistance
- Used as an adjuvant to other first line drugs

SGLT-2 INHIBITORS:

- SGLT-2 causes re-absorption of glucose in proximal tubules of kidney
- Inhibition of SGLT 2 causes glucosuria and lowers blood glucose level

Adverse Effects: Urinary infections, electrolyte imbalances.

INSULIN

Decreases blood glucose by

- Increasing entry of glucose in muscle and fat
- Inhibiting glycogenolysis
- Increasing glycolysis

PREPARATIONS:

- Conventional preparations
    - Obtained from pork and beef
    - Less costly
    - Produces allergic reactions
- Human insulin
    - Prepared from recombinant DNA technology
    - Allergic reactions are rare
    - Costlier
- Nasal insulin
    - Powdered form of recombinant human insulin
    - Delivered through an inhaler in to lungs
    - Helps in avoiding daily injections but costly.

TYPES OF INSULINS

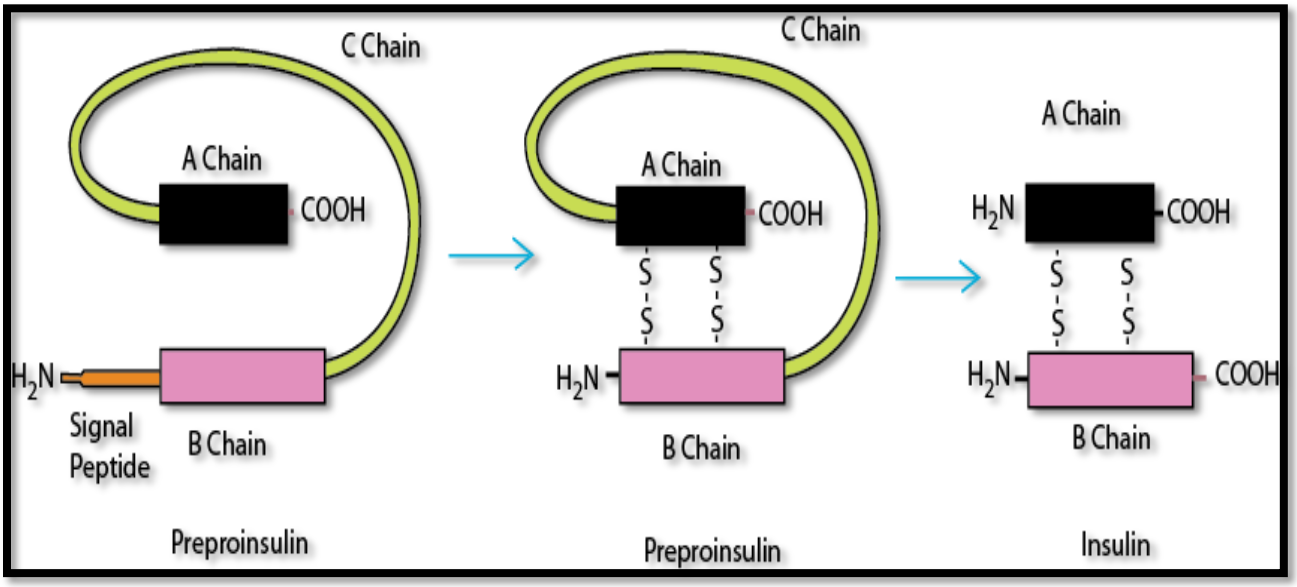
- Ultra short acting
    - Lispro - Glulisine - Aspart
- Short acting
    - Regular - Semi lente insulin
- Intermediate acting
    - Lente - Neutral protamine hagedron
- Long acting
    - Ultra lente - Protamine zinc

INDICATIONS OF INSULIN THERAPY

- All cases of Type 1 diabetes
- Type 2 diabetes
    - Not controlled on oral hypoglycaemic drugs
    - In pregnancy
    - Complications like DKA and Hyperosmolar hyperglycaemic state
    - To tide over stressful conditions like infection and surgery.

INSULIN[24]

- Insulin is a peptide hormone secreted by beta cells of the pancreas.
- Insulin is first synthesized as a single polypeptide chain called preproinsulin (110 amino acid), which is the processed to proinsulin and finally cleaved to form insulin and C-peptide.
- Insulin (51 amino acids) has two, A(21 a.a) and B (30 a.a) chains linked by disulphide linkages.
- Equimolar concentrations of insulin and C-peptide are secreted with insulin having a half life of 5-6 minutes and C-peptide a half life of 30 minutes.



STIMULUS FOR SECRETION [25]

- Insulin secretion is an effectively regulated process designed to provide a stable concentration of glucose in blood during both fasting and fed state.
- Factors which increase the release of insulin are:

    - Carbohydrate rich food : increases blood glucose levels which is the most important stimulus for insulin secretion. Glucokinase in liver and pancreatic β cells converts blood glucose to glucose 6- phosphate. In β cells of pancreas it functions to detect high concentrations of glucose, converting it to glucose 6 phosphate which stimulates insulin secretion.

    - Amino acids

    - Fatty acids

    - Ketone bodies

    - GI hormones : also called incretins eg: Glucagon like peptide-1 (GLP-1),Gastric- inhibiting polypeptide (GIP), cholecystokinin

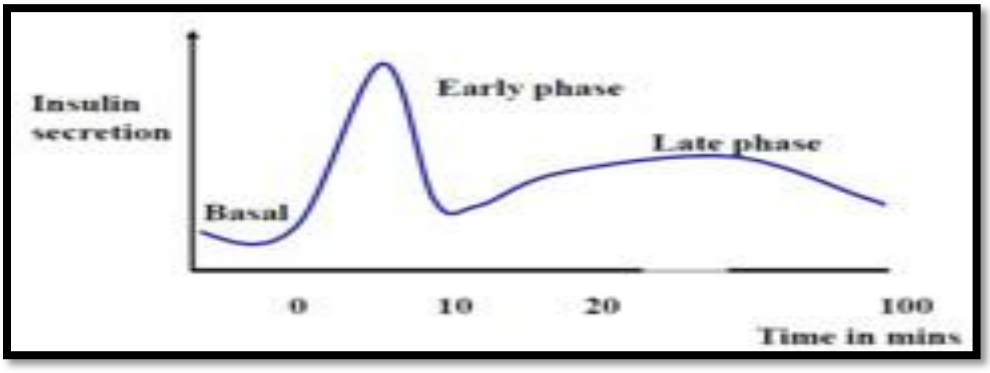
INHIBITORS OF SECRETION [25]

- Decreased blood glucose or hypoglycemia

- Stress, severe exercise, trauma : due to activation of the sympathetic nervous system and release of epinephrine.
- Hormones : Somatostatin reduces the insulin secretion
- Excessive gluconeogenesis.

BIPHASIC RESPONSE

- Insulin secretion to glucose is a biphasic response.
- In the normal post-absorptive period, low basal levels of circulating insulin are maintained through constant β-cell stimulation. This suppresses lipolysis, proteolysis, and glycogenolysis.
- A burst of insulin secretion occurs within few minutes of ingesting a meal, in response to transient increases in the levels of circulating glucose and amino acids. This lasts for up to 15 minutes and is followed by the postprandial secretion of insulin.[22]



MECHANISM OF ACTION:[26]

- Insulin action is mediated through insulin receptors which belong to the tyrosine kinase family.
- Insulin receptor has 2 extracellular α and 2 transmembrane β subunits.
- The α subunit binds with insulin while the β subunit gets phosphorylated and mediates tyrosine kinase activity.
- Activation of this receptor initiates phosphorylation of other intracellular proteins like Insulin Receptor Substrates (IRS) which interact with effectors and amplify the signaling process.
- This plays an important role in Glucose disposal after meal ingestion.

ACTIONS OF INSULIN[27]

a) CARBOHYDRATE METABOLISM:

- Adipose tissue

  -↑ Glucose uptake (via upregulation of GLUT-4 receptors)

- Muscle

  -↑ Glucose uptake (via upregulation of GLUT-4 receptors)

  -↑Glycogen synthesis (activated Glycogen synthase enzyme)

- Liver

-↓Glucose output due to ↓ gluconeogenesis, increased glycogen synthesis and increased glycolysis.

b) PROTEIN METABOLISM:

- Muscle

  -↑Amino acid uptake and protein synthesis in ribosomes

  -↓protein catabolism

  -↓release of gluconeogenic amino acids

- Liver

  -↑protein synthesis

c) LIPID METABOLISM:

- Adipose tissue

  ↑fatty acid synthesis

  -↑triglyceride deposition

  -inhibition of hormone sensitive lipase

- Muscle

  -↑ketone uptake

- Liver

  -↓ketogenesis

  -↑lipid synthesis.

ANTI INSULIN HORMONES [28]

1. Pancreas
    - Glucagon
    - Somatostatin (from delta cells) – inhibit both insulin and glucagon but more prominent effect on insulin- hyperglycemia

2. Adrenal medulla
    - Epinephrine

3. Adrenal cortex
    - Glucocorticoids: stimulates gluconeogenesis and ↓ utilization of glucose by extra hepatic tissues

4. Anterior pituitary gland
    - Growth hormone: Promotes gluconeogenesis and glycogenolysis in liver and decreases glucose utilization by muscles

    - ACTH - Stimulates production of steroids in adrenal cortex and enhanced release of cortisol.

GLUCAGON[28]

- Glucagon is a polypeptide hormone secreted by the alpha cells of the pancreas.

- It is composed of 29 amino acids that are arranged in a single polypeptide chain.

- Glucagon is synthesized as a large precursor molecule (preproglucagon) which is converted to glucagon through a series of proteolytic cleavages similar to insulin biosynthesis.

Physiology of glucagon [27,29]

Glucagon is secreted by the alpha cells of pancreas directly into the portal vein and carried to the liver. It has a circulating half life of 5-10 mins and is degraded primarily by the liver.

The most important physiologic action of glucagon occurs during the postabsorptive and fasting states. Glucagon stimulates glycogenolysis, gluconeogenesis, and ketogenesis by the liver, and lipolysis in adipose tissue.

In states of low blood glucose, the central nervous system triggers neural–sympatho-adrenal hormones to counteract this hypoglycemia. The **ventromedial hypothalamus** is an important sensor of hypoglycemia and initiates certain neural afferent signals which stimulate counter-regulatory responses by way of secretion of catecholamines, glucagon, growth hormone, and glucocorticoids.

Stimulus for glucagon secretion[25]

1. Low blood glucose: A reduction in the plasma glucose level is the main stimulus for glucagon release. During prolonged or overnight fasting, elevated levels of glucagon prevent the development of hypoglycemia.

2. Amino acids: Amino acids from meals rich in proteins stimulate the release of both glucagon and insulin. The glucagon prevents the hypoglycemia which would otherwise occur due to increased insulin release following a protein rich meal.

3.Epinephrine: Stimulation of the sympathetic nervous system causes increased levels of circulating epinephrine (by adrenal medulla), and/or norepinephrine both of which enhance glucagon release. During periods of stress or severe exercise, the elevated

epinephrine levels can increase glucagon secretion regardless of the blood glucose concentration.

Inhibitors of glucagon secretion[25]

1. Carbohydrate rich meal results in elevation of blood glucose level. This inhibits the release of glucagon
2. Insulin is released when blood glucose level rises. Insulin inhibits the release of glucagon.
3. Somatostatin: is a hormone produced from delta cells of pancreas. It inhibits release of both insulin and glucagon but its action on insulin is more marked.
4. Glucagon like peptide 1(GLP-1): is an incretin released from the gut in response to oral glucose. It inhibits glucagon release by acting on GLP-1 receptors on the alpha cells of pancreas.

Effects of glucagon[25,29]

1.Effects on carbohydrate metabolism: Glucagon causes an enhanced breakdown of liver (not muscle) glycogen, resulting in an immediate rise in blood glucose levels.

Glucagon also inhibits the enzyme pyruvate kinase of the glycolytic pathway due to which PEP (phospho enol pyruvate) is unable to continue in glycolysis and enters the gluconeogenesis pathway instead. This causes inhibition of hepatic glycolysis and stimulatation of gluconeogenesis by glucagon.

2.Effects on lipid metabolism: Glucagon activates lipolysis in adipose tissues releasing free fatty acids into circulation. These are taken up by liver and oxidized to acetyl coenzyme A, excess of which is diverted to ketone bodies synthesis.

3.Effects on protein metabolism: Glucagon increases the hepatic uptake of amino acids, which are subsequently used as substrates for gluconeogenesis.

4.Effect on Red blood cells . The mature erythrocyte lacks mitochondria and is completely dependent on glycolysis for ATP production. This ATP is required to meet the metabolic needs of the RBC and for maintaining the flexible biconcave shape of the RBC, which allows it to squeeze through narrow capillaries.[25]

The final step in glycolysis is the synthesis of pyruvate from phosphoenolpyruvate catalyzed by the enzyme **pyruvate kinase.**

This enzyme is hormonally activated by insulin and inhibited by glucagon via the

c-AMP pathway. Inhibition of pyruvate kinase by glucagon leads to a deficiency of ATP. ATP is necessary for the maintenance of the normal biconcave disc shape of the RBC which allows it to squeeze through narrow capillaries. Alterations in the shape lead to poor red cell deformability and early phagocytosis by cells of the reticulo-endothelial system, particularly the spleen. [25]

This premature lysis of red blood cells results in **hemolytic anemia.**

ROLE OF GLUCAGON IN INSULIN RESISTANCE

Glucagon opposes the actions of insulin (**physiological antagonist**) in the following ways contributing to insulin resistance:[25]

A) PROMOTES GLUCONEOGENESIS by 3 mechanisms.

Gluconeogenesis is the process of synthesizing glucose or glycogen from non carbohydrate precursors like glucogenic amino acids, lactate, glycerol and propionate.

The major tissues for gluconeogenesis are **liver and kidney** with kidney contributing upto 40% of gluconeogenesis in starvation.

Excess gluconeogenesis occurs in critically ill patients in response to injury, infection and stress causing hyperglycemia which causes changes in osmolarity of body fluids, reduced blood flow, intracellular acidosis and production of superoxide radicals. Excessive gluconeogenesis contributes to hyperglycemia in type 2 diabetes because of impaired sensitivity of gluconeogenesis to insulin control.

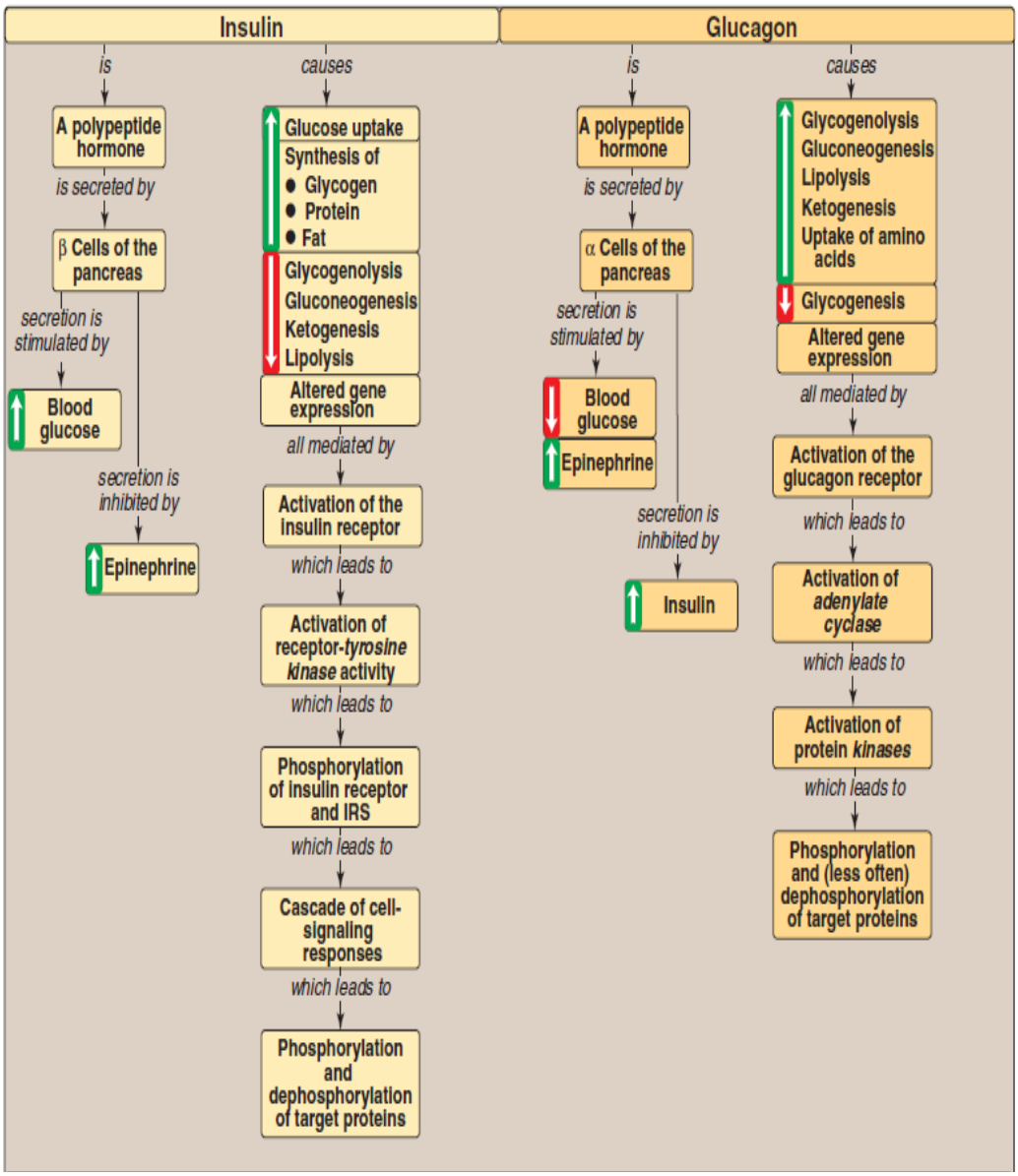
*** Changes in allosteric effectors:** Glucagon lowers the level of fructose 2,6-bisphosphate and inhibits phosphofructokinase-1, thus favoring gluconeogenesis over glycolysis.

***Covalent modification of enzyme activity:** Glucagon elevates intracellular cyclic AMP (cAMP) levels and activating cAMP-dependent protein kinase which convert pyruvate kinase to its inactive (phosphorylated) form. This decreases the conversion of Phospho enol pyruvate (PEP) to pyruvate thus diverting it to the synthesis of glucose.

***Induction of enzyme synthesis:** Glucagon increases the transcription of the gene for PEP-carboxykinase, thereby increasing the availability of this enzyme as levels of its substrate rise during fasting.

B) PROMOTES GLYCOGENOLYSIS: Glycogen causes increased glycogen degradation in the liver through covalent modification (phosphorylation) and activation of glycogen phosphorylase enzyme.

C) <u>PROMOTES LIPOLYSIS AND KETOGENESIS</u> : by activation of hormone sensitive lipase and inactivation of acetyl CoA carboxylase by covalent modifications. The glycerol thus produced is used as a substrate for gluconeogenesis and the free fatty acids a source of ketone bodies.



EPINEPHRINE[30]

- Also known as Adrenaline (Adr), is a hormone produced by the adrenal medulla in response to sympathetic stimulation.
- It is known as the hormone for "flight or fight" response.
- It has α1,α2,β1,β2 and weak β3 action.
- **Stimulus for release** include: stress, trauma, cold, exercise and low blood glucose levels.

ACTIONS [30]:

1. **Heart:** Adrenaline causes β1 mediated increase in heart rate, force of contraction. Cardiac output and oxygen consumption increase markedly.
2. **Blood vessels:** Vasoconstriction(α) predominates in muco-cutaneous and renal beds. Vasodilatation (β2) predominates in skeletal muscles, liver and coronaries.
3. **Blood pressure:** slow i.v or s.c injection produces a biphasic response of increase in systolic and fall in diastolic BP, as β receptors are more sensitive than α receptors.
4. **Respiration :** Adr is a potent bronchodilator (β2) and decongests the bronchial mucosa (α).

5. **Eye:** causes mydriasis by contraction of α1 receptors in the dilator papillae. Reduces intra ocular tension.
6. **Skeletal muscle:** facilitates neuromuscular transmission by release of acetylcholine due to α receptor activation. Increases blood supply to muscle by β2 mediated vasodilatation.
7. **Metabolic effects:** opposes the actions and release of insulin

- Increases blood glucose levels by
    - Inhibition of glycogenesis
    - Stimulation of glycogenolysis in liver (activates glycogen phosphorylase enzyme by phosphorylation via c-AMP dependant protein kinases)
    - Reduces the peripheral uptake of glucose in insulin-sensitive tissues
    - Stimulates gluconeogenesis – by increasing availability of substrates
- Promotes Lipolysis:
    - By phosphorylation and inactivation of acetyl CoA carboxylase enzyme necessary for fatty acid synthesis.
    - By activation of hormone sensitive lipase (in adipose tissues), it stimulates the breakdown of tri acyl glycerol (TAG) resulting in increased plasma glycerol and FFA which are used as substrates for gluconeogenesis and as a source of free radicals respectively.

- **Inhibits insulin release** by acting on the alpha2 receptors on beta cells of pancreas.

- **Increases glucagon secretion,** irrespective of blood glucose levels in times of stress by activating the β2 receptors on the alpha cells of the pancreas.
- All these actions mediated by epinephrine supplement the effect of glucagon and antagonize the effects of insulin thus promoting a state of insulin resistance.

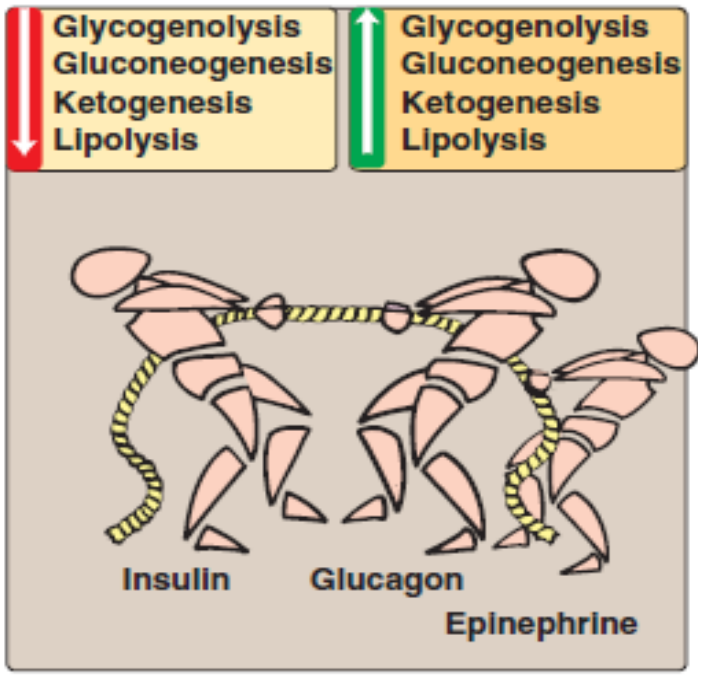


Figure 23.10
Opposing actions of insulin and glucagon plus epinephrine.

HYPOGLYCEMIA[25]

Regular supply of glucose is necessary for tissues like Nervous system and Erythrocytes. Hypoglycemia is a medical emergency as transient hypoglycemia can cause cerebral dysfunction, while severe and prolonged hypoglycemia can cause brain death.

The most important hormones to combat hypoglycemia are elevated epinephrine and glucagon along with diminished release of insulin.

PHYSIOLOGICAL HORMONAL RESPONSES TO HYPOGLYCEMIA

Humans have two overlapping glucose-regulating systems activated by hypoglycemia:

1) the alpha cells of pancreas which release glucagon; and
2) receptors in the hypothalamus, which respond to abnormally low blood glucose levels.

The hypothalamic glucoreceptors triggers the secretion of epinephrine (mediated by the sympathetic nervous system) and the release of adrenocorticotropic hormone (ACTH) and growth hormone by the anterior pituitary.

Glucagon, epinephrine, cortisol, and growth hormones are sometimes called the "**counter-regulatory**" hormones because each opposes the action of insulin on glucose utilization.

1. **Glucagon and epinephrine:** are most important in the acute or short term regulation of blood glucose levels. Glucagon stimulates hepatic glycogenolysis and gluconeogenesis. Epinephrine promotes glycogenolysis and lipolysis, inhibits insulin secretion, and inhibits the insulin-mediated uptake of glucose by peripheral tissues.

2. **Cortisol and growth hormone:** play a role in the long-term management of blood glucose metabolism.

SYMPTOMS OF HYPOGLYCEMIA

The symptoms of hypoglycemia can be divided into two categories.

a) **Adrenergic symptoms**—anxiety, palpitation, sweating, tremors - mediated by epinephrine release regulated by the hypothalamus in response to hypoglycemia. Usually occurs when the blood glucose falls abruptly.

b) **Neuroglycopenic symptoms** (due to impaired delivery of glucose to the brain - impairment of brain function) - headache, confusion, slurred speech, seizures, coma, and death. These often result from a gradual decline in blood glucose which deprives the brain of fuel, but fails to trigger an adequate epinephrine response.[25]

CONSEQUENCES OF RECURRENT HYPOGLYCEMIC ATTACKS

- Hypoglycaemia - associated autonomic failure
- Higher cardiovascular mortality
- Impairment of cognitive function especially in children
- Chronic mood disorders like depression and anxiety

INSULIN RESISTANCE

Insulin resistance refers to suboptimal response of body tissues, especially liver, skeletal muscle and fat to physiological amounts of insulin.[30]

"Hyperinsulinemia" is the classic indicator of insulin resistance.[31]

Insulin resistance plays an important role in pathogenesis and complications of Type 2 DM.

CONTRIBUTORS TO INSULIN RESISTANCE

1. Anti insulin hormones: (Diabetogenic hormones) – glucagon, epinephrine, GH, cortisol

2. Hyperinsulinemia: repeated stimulation leads to the downregulation of insulin receptors (due to enhanced internalization and degradation of the receptor-insulin complex).[31]

Hyperinsulinemia has also been shown to downregulate insulin-receptor substrates, producing an even greater reduction in insulin signaling.

3. Stress : acts in multiple ways to promote insulin resistance. The various mechanisms include:

- Stimulation of the sympathetic nervous system - release of epinephrine from the adrenal medulla and glucagon from the alpha cells of pancreas – both oppose insulin action.[25]

- During stress, there is an increase in free radical (ROS) production due to high respiratory oxygen intake and metabolic turnover – producing a state of Oxidative stress.[32]

4.Inflammatory Cytokines: Diabetes is considered as a state of ongoing subclinical inflammation. A number of inflammatory cytokines are elevated in diabetes specifically TNF- α, IL-6 and IL-1 and Isoprostanes.

The various mechanisms though which inflammatory cytokines mediate insulin resistance are [33]:

- inhibition of signaling downstream of the insulin receptor.
- Induction of iNOS , overproduction of which impairs insulin action on muscle and beta cell function.

TNF-α inhibits phosphorylation of serine residues of IRS-1 in response to insulin. It reduces IRS-1 binding to insulin receptor, thereby inhibiting downstream signaling and insulin action.

IL-6 stimulates C-reactive protein which is positively correlated with insulin resistance, obesity, and endothelial dysfunction.

IL-1 inhibits insulin secretion by pancreatic beta cells.

Isoprostanes: are prostaglandin (PG) – like substances produced in vivo by non-ezymatic, free radical catalyzed peroxidation of arachidonic acid. The best characterized among these Isoprostanes are the F_2 isoprostanes particulary **8-iso- $PGF_2\alpha$.** Measurement of Isoprostanes in urine and plasma is considered as a sensitive and reliable tool for identifying oxidative stress in vivo.

Isoprostanes are not just biomarkers of oxidative stress but have many biological effects, suggesting that they may have a role as pathophysiologic mediators of oxidant injury. They mediate their biological actions by acting on prostanoid receptors. **Actions include** [34]**:**

- Induce inflammation and promote atherogenesis through activation of MAP kinases .
- Promote platelet activation ,
- Induce mitogenesis in vascular smooth muscle cells ,
- Stimulate fibroblast proliferation , and
- Alter endothelial cell biology by increasing expression of endothelin-1.

- Vasoconstriction by inducing synthesis of thromboxane in the endothelium.

PG-$F_2\alpha$ has also been shown to induce membrane damage in RBCs resulting in abnormal red cell morphology (crenated cells and spherocytes) and subsequent hemolysis, which were effectively reversed by Vitamin E, a potent antioxidant.[35]

5.<u>Oxidative stress and ROS</u>:[31]

Chronic hyperglycemia leads to increased production of free radicals from the mitochondria and Endoplasmic reticulum (ER stress) resulting in oxidative stress. This tilts the normal balance between oxidant and antioxidants to favor oxidative damage.

Oxidative stress ⟶ activation of serine kinases ⟶ ↑ serine phosphorylation of the insulin receptor and its substrates ⟶ decreased tyrosine phosphorylation of the receptor substrates ⟶ altered second messenger pathway ⟶ decreased translocation of glucose transporter (GLUT4) ⟶ **INSULIN RESISTANCE**

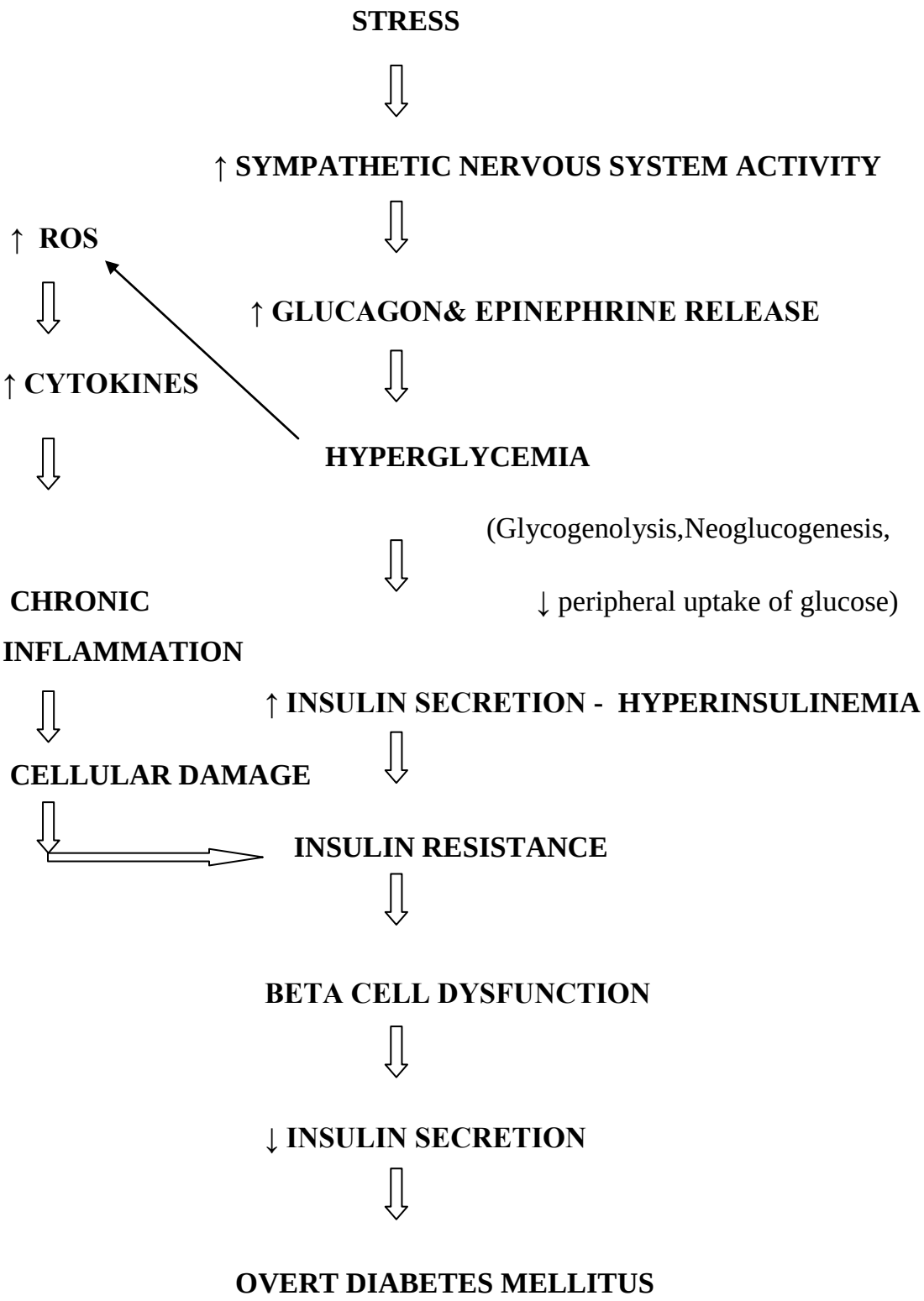
SITES OF INSULIN RESISTANCE [31]

A) SKELETAL MUSCLE: Due to reduced insulin receptor substrate-1 (IRS-1)–associated tyrosine phosphorylation and 1-phosphatidylinositol 3-kinase (PI 3-kinase) activity in skeletal muscle.

B) ADIPOSE TISSUE: Proinflammatory adipokines induce systemic insulin resistance by direct effects on insulin signaling, downregulation of genes needed for normal insulin action and negative regulation of PPARα.

C) LIVER: due to an intrinsic abnormality of insulin signaling in the hepatocyte.

D) HYPOTHALAMUS : Mutation in the hypothalamic LepR-b receptor or decrease in circulating levels of leptin has been associated with reduced peripheral insulin sensitivity and a state of insulin resistance.[36]


STRESS
↑ SYMPATHETIC NERVOUS SYSTEM ACTIVITY
↑ ROS
↑ GLUCAGON& EPINEPHRINE RELEASE
↑ CYTOKINES
HYPERGLYCEMIA
(Glycogenolysis,Neoglucogenesis,
↓ peripheral uptake of glucose)
CHRONIC INFLAMMATION
↑ INSULIN SECRETION - HYPERINSULINEMIA
CELLULAR DAMAGE
INSULIN RESISTANCE
BETA CELL DYSFUNCTION
↓ INSULIN SECRETION
OVERT DIABETES MELLITUS

OXIDATIVE STRESS

Biological free radicals are highly unstable molecules which are products of normal cellular metabolism. Having unpaired electrons they react with various organic substrates such as lipids, proteins and deoxyribonucleic acid (DNA) and damage cell structures leading to Oxidative stress.[37]

TYPES OF FREE RADICALS:[38]

1.Reactive oxygen species:

- Superoxide anion radical (O2-) - Hydrogen peroxide(H2O2-) - -
- Hydroxyl radical (OH-) - Peroxy radical (ROO)
- Hypochlorous acid (HOCl)

2.Reactive Nitrogen species :

- Peroxynitrite (ONOO-) - Nitric oxide ($NO^{\cdot}$)

Free radicals produced under physiological conditions are maintained at steady state levels by endogenous or exogenous antioxidants which act as free radical scavengers. However, when the production of free radicals overwhelms the detoxification capacity of the cellular antioxidant system, **oxidative stress** occurs causing biological damage.[39,40]

OXIDATIVE STRESS IN DIABETES

In diabetics, there is a significant increase in the production of free radicals. This results in an imbalance between the oxidants and antioxidants resulting in **oxidative stress** that leads to activation of stress-sensitive intracellular signaling pathways and formation of gene products that cause cellular damage and result in various diabetic complications.[41,42.]

Hyperglycemia is also directly additive to oxidative stress. Glucose can autoxidize, generating free radicals like hydrogen peroxide and reactive ketoaldehydes.[6]

Oxidative stress in diabetes can cause:

1. **Increased insulin resistance**

Elevated glucose and free fatty acids levels in diabetes stimulate the production of free radicals.

These free radicals inhibit the normal tyrosine kinase pathway resulting in insulin resistance.

Oxidative stress ⟶ activation of serine kinases ⟶ ↑ serine phosphorylation of the insulin receptor and its substrates ⟶ ↓ tyrosine phosphorylation of the receptor substrates ⟶ altered second messenger pathway ⟶ ↓ translocation of glucose transporter (GLUT4) ⟶ **INSULIN RESISTANCE.**[43]

2. **Beta cell failure**

β-Cells are particularly susceptible to oxidative stress as they have intrinsically lower concentrations of antioxidant enzymes.[44]

Excess free radical generation in diabetes further adds to this stress leading to beta cell failure.

↑ ROS/RNS ⟶ impaired insulin signaling ⟶ insulin resistance ⟶ ↑ demand on β- cells to secrete insulin ⟶ β- cell failure

3. **Impaired vasodilatation**

Oxidative stress is implicated as a cause for abnormal endothelial mediated relaxation of blood vessels in diabetes. Experimental studies have shown that ROS inactivate endothelium-derived relaxing factor or nitric oxide (NO) and impair endothelium-dependent relaxation.[6]

SOURCES OF FREE RADICALS AND ASSOCIATED COMPLICATIONS IN DIABETES

1. SORBITOL (POLYOL) PATHWAY [45,47]:

Under normal physiological conditions, **Aldose reductase** reduces glucose to sorbitol using cellular NADPH for the reaction. This enzyme is found in lens, retina, Schwann cells of peripheral nerves, kidney, liver, placenta and RBCs. In liver there is a second enzyme called **sorbitol dehydrogenase**, which oxidizes sorbitol to fructose.

In diabetes, excess glucose enters into these cells (as they do not require insulin for uptake of glucose), resulting in increased production of sorbitol which becomes trapped inside the cell. This is exaggerated in cells where levels of sorbitol dehydrogenase is low

or absent like lens, retina, kidney, and nerves. As a result, sorbitol accumulates within these cells, producing strong osmotic effects resulting in water retention and cellular swelling. This in part explains the pathogenesis of **cataract formation, retinopathy, neuropathy, and nephropathy** seen in diabetics.

Excess sorbitol production depletes cellular NADPH which is required to maintain the primary intracellular antioxidant, glutathione, in its reduced state [1], thus predisposing to oxidative stress.

2. ADVANCED GLYCATION END PRODUCTS (AGEs)

When the blood glucose level is consistently elevated, there is an increase in non-enzymatic attachment of glucose to amino groups of proteins leading to the formation of Advanced Glycation End Products (AGEs). Once formed, AGEs can cause tissue damage by **two main mechanisms**:

(1) Formation of cross links that alter protein structure and function.

(2) Interaction of AGE with AGE receptors on the surfaces of various cells such as endothelial cells, macrophages, neurons, and smooth-muscle cells results in the activation of cell signaling and gene expression that induce oxidative stress and inflammation.[46,47]

In diabetes, AGEs have been implicated in the pathogenesis of retinopathy, nephropathy, atherosclerosis, cardiomyopathy, diastolic dysfunction and systolic hypertension.[48]

3. PROTEIN KINASE C (PKC) ACTIVATION [49,50]

Protein kinases such as PKA, PKC are intra cellular signaling molecules. They phosphorylate serine and threonine residues in target proteins.Physiologically the most important activator of PKC is Diacylglycerol (DAG)

In diabetes, hyperglycemia causes the activation of PKC by two major pathways:

- Enhanced *de novo* synthesis of diacylglycerol (DAG) from glucose.
- Interaction between AGE's and their cell-surface receptors can result in enhanced activity of certain PKC isoforms .

PKC seems to regulate diabetic complications on multiple levels by activation of NADPH oxidase, phospholipase A2, endothelin-1, Vascular endothelial growth factor, Transforming growth factor-β, and NF-KB. It also inhibits NO synthesis by inhibiting NO synthase enzyme.

Thus activation of PKC is related to hyperplasia of smooth muscle cells, vasoconstriction and enhanced synthesis of extracellular matrix proteins which play an important role in the onset and progression of vascular dysfunction in diabetes.[51,52]

RED BLOOD CELL

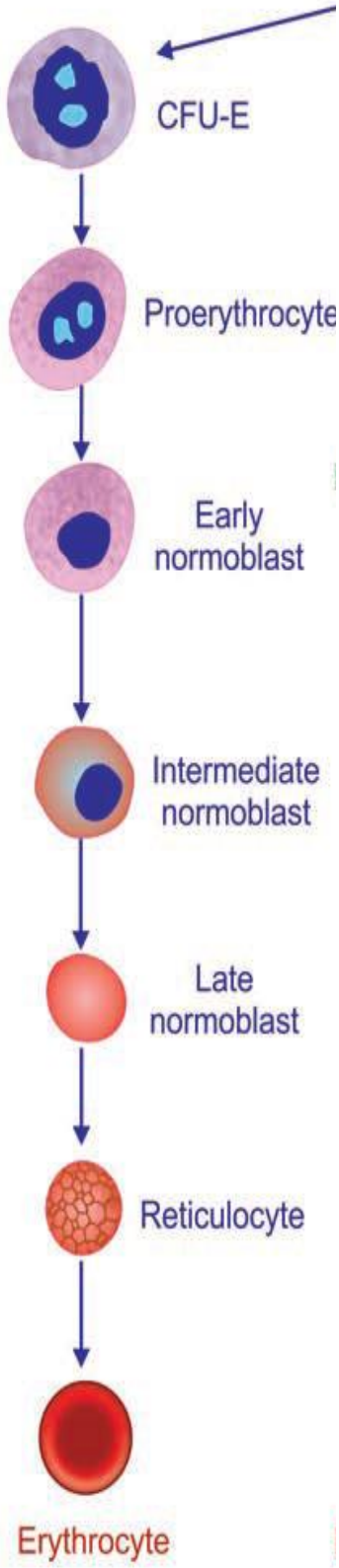
Red blood cells (RBCs) or Erythrocytes are formed within the bone marrow from Pluripotent hemopoietic stem cells. Their red color is due to the presence of the coloring pigment called hemoglobin.

The process of the origin, development and maturation of erythrocytes is known as **Erythropoiesis**.[53]

STAGES OF ERYTHROPOIESIS [54]

Pluripotent hemopoietic stem cells differentiate into Colony forming units- Erythrocyte (CFU-E), which then pass through the following stages to form the mature red blood cell.

PLURIPOTENT HEMOPOIETIC STEM CELLS



- **Proerythroblas**t: first cell derived from CFU–E. Contains large nucleus with nucleoli. ***Hemoglobin synthesis starts***. Size 20 microns.

- **Early Normoblast**: ***nucleoli disappear*** and condensation of chromatin occurs. Size 15 microns.

- **Intermediate Normoblast**: chromatin shows further condensation and ***hemoglobin starts appearing***. Size is 10 -12 microns.

- **Late Normoblast**: ***Nucleus disintegrates*** and ***disappears*** (a process known as Pyknosis). Hemoglobin quantity increases. Size 8-10 microns

- **Reticulocyte**: also known as immature RBC. Cytoplasm contains the ***reticular network*** formed by remnants of disintegrated organelles (golgi apparatus, mitochondria etc). During this stage, the ***cells enter circulation by diapedesis***.

- **Matured Erythrocyte**: ***reticulum disappears*** and cells attain a ***biconcave shape***. Size 7-8 microns.

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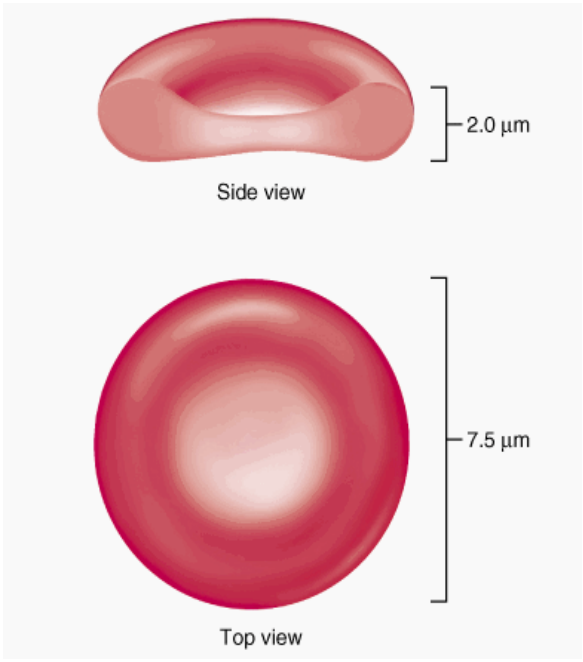

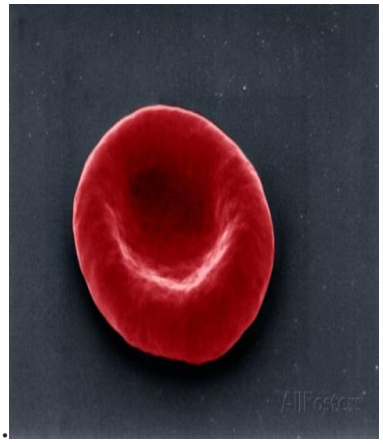

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Average RBC count is 5.4 million/µL in men and 4.8 million/µL in women. [55] Their main function is to transport oxygen from lungs to tissues and remove carbon dioxide from the tissues (in the form of bicarbonate ion).[56]

Normal life span of RBC is about 120 days.

Main site of destruction is in the reticulo-endothelial system of the liver and spleen, particularly the macrophages of spleen.[57]

STRUCTURE OF RBC

The red blood cell membrane has three basic components, lipid bilayer, transmembrane (integral) proteins and a cytoskeletal network.

The lipid bilayer is made up of 60% phospholipids, 30% cholesterol and 10% glycolipids. Cholesterol provides flexibility and stability to the red cell membrane.

The sub-membrane cytoskeleton of the RBC consists of several proteins like spectrin, ankyrin, protein 4.1 and actin that form a quasi-two-dimentional meshwork under the lipid layer. Its biconcave shape is maintained by these proteins especially the spectrin network and the lipid bilayer.[12] This characteristic shape allows it to squeeze through narrow splenic capillaries without getting damaged.

RBCs lack a nucleus (no DNA) and organelles like mitochondria, ribosome and endoplasmic reticulum.[58] Its cytoplasm contains haemoglobin (major protein), enzymes for glycolytic (eg:pyruvate kinase) and HMP shunt (eg:G6PDenzyme) pathway and endogenous antioxidants like reduced glutathione, Vitamin C and Vitamin E.

ERTHROCYTE METABOLISM

Glycolysis is the only source of ATP production in a mature RBC.[59]

Erythrocytes lack mitochondria, therefore glycolysis always terminates in lactate formation as the subsequent reactions for pyruvate oxidation are absent.[57]

Glucose entry into RBCs is independent of insulin. Glucose enters RBCs by Na^+-independent facilitated diffusion via GLUT-1.

90% of the entered glucose enters glycolysis for **ATP** production and the remaining **10%** glucose enters into the hexose monophosphate (HMP) shunt pathway to generate **NADPH** using G6PD (glucose 6-phosphate dehydrogenase) enzyme. This NADPH is used by RBCs to reduce glutathione, its primary intracellular antioxidant.[12]

REASONS FOR HEMOLYTIC ANEMIA IN DIABETES

Diabetes is a state of excess free radical production. Chronic stress plays a critical role, and along with hyperglycemia, they increase the generation of Reactive oxygen species (ROS) from mitochondria and endoplasmic reticulum leading to Oxidative stress.[60,61]

In diabetes there is an increased flux of glucose through the sorbitol pathway which results in the depletion of NADPH. This NADPH is essential in RBCs for regenerating reduced glutathione (GSH), the prime antioxidant protecting the RBCs from oxidative

damage and necessary for maintaining the cellular glutathione pool. Furthermore, ROS induced cell membrane damage allows the available GSH to pass through the membrane causing depletion of GSH in the cytoplasm of the RBC. As the mature erythrocyte lacks a nucleus and ribosome it cannot regenerate GSH or synthesize new protein, thus making it vulnerable to oxidative damage.[62]

The free radicals generated in diabetes stimulate the release of a number of inflammatory cytokines like TNF-alpha, IL-1 & 6 and Isoprostanes ($PG\text{-}F_2\alpha$).[35] These cytokines further increase the generation of ROS, a viscious cycle results, worsening oxidative damage to cells.

Red blood cells are especially prone to this oxidative damage as they contain high levels of hemoglobin and oxygen and are the first cells to be affected by adverse conditions. Since RBCs lack mitochondria, glycolysis is the only way of generating ATP which is required to maintain its characteristic biconcave shape and flexibility. In the absence of adequate ATP, the RBC membrane loses integrity and is prone to lysis.[63]

Glucagon plays an important role in the pathogenesis of diabetes. Stress activates the sympathetic nervous system which enhances the release of glucagon. Glucagon causes hyperglycemia which in turn enhances free radical generation and oxidative stress. Glucagon also inactivates Pyurvate kinase, an enzyme of the glycolytic pathway, thereby

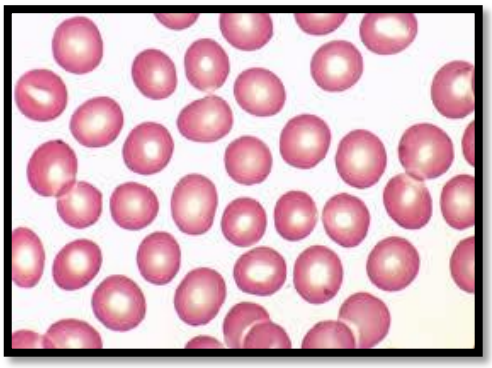
reducing production of ATP.[63] Lack of sufficient ATP and free radical mediated injury damages RBC membrane, increasing its fragility and leading to hemolytic anemia.

ROS induced oxidation of membrane proteins make red cells rigid and less deformable, allowing them to be removed by the macrophages in the reticuloendothelial cells of spleen. Oxidation of sulphhydryl groups in hemoglobin leads to the formation of disulfide cross-linkages between adjacent globin chains causing a distortion of hemoglobin structure and forming denatured visible precipitates called "**Heinz bodies"** which attach to the red cell membrane and excised by the macrophages of the spleen.[62]

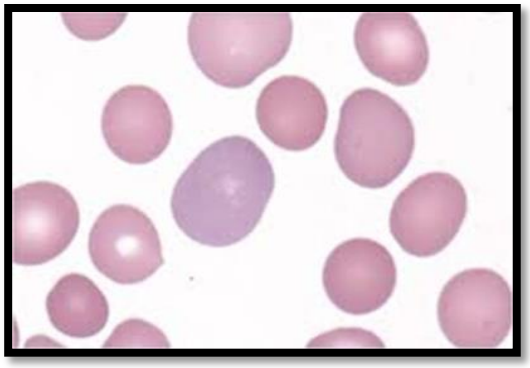
Structural changes in the RBC due to oxidative damage include:

1. **Irregularly contracted and Crenated cells** – Due to altered cell membrane integrity[64]
2. **Reticulocytes** – immediate precursors of mature red blood cells containing a reticulum(made of disintegrated cell organelles).[54]
3. **Heinz bodies** – insoluble aggregates of oxidized haemoglobin [65]
4. **Bite cells** - Remaining cells after removal of Heinz bodies by spleen [66]
5. **Spherocytes -** Smaller and denser than normal RBC with spheroidal structure[67]

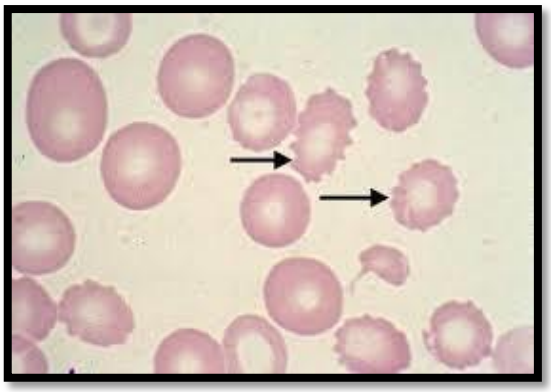
NORMAL RBC



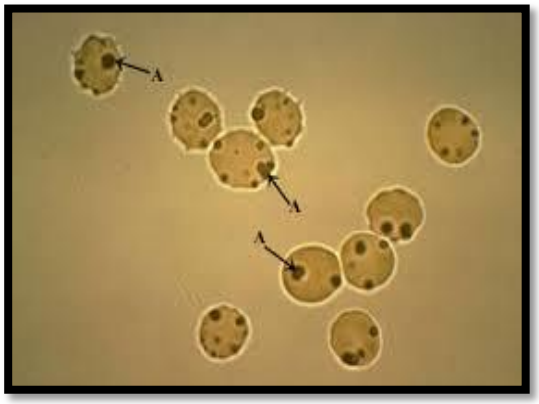
RETICULOCYTES



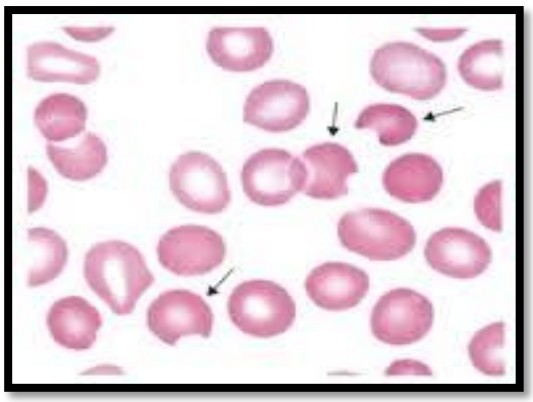
CRENATED RBCs



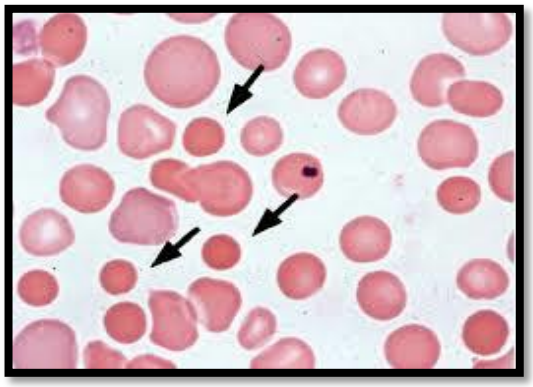
HEINZ BODIES



BITE CELLS



SPHEROCYTES



ANTIOXIDANTS

DEFINITION:

An antioxidant is a stable molecule that interacts and neutralizes free radicals preventing them from causing tissue damage.[68]

CLASSIFICATION

The various **Antioxidants** are categorised into [44]:

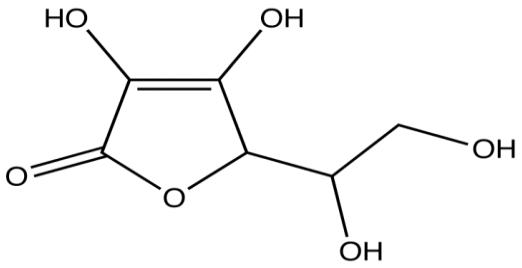
a) **Enzymatic antioxidants**: Glutathione reductase, Superoxide dismutase, Glutathione peroxidase, Catalase.

b) **Non-enzymatic antioxidants**: α lipoic acid, Vitamins C and E, bioflavinoids coenzyme Q , antioxidant minerals (copper ,selenium, zinc and manganese).

ASCORBIC ACID (VITAMIN C)

It is a water soluble vitamin and the major antioxidant in plasma and tissues.

STRUCTURE:



ACTIVE FORM:

- Dehydroascorbic acid (DHA)
- Ascorbic acid (AA) [69]

DIETARY SOURCES:

PLANT SOURCES:

-Indian goose berry - Chilli pepper - Guava - Black currant

- Red pepper - Lemon -Orange - Broccoli

ANIMAL SOURCES: - Lamb brain - Calf liver - Chicken liver, kidney[69]

BIOSYNTHESIS

-Vitamin C is an **essential nutrient** for human beings as they lack an enzyme required for its synthesis.[69]

-It is not stored in any organ in the body.

-Plants and animals can synthesize their own vitamin C.

PHARMACOKINETICS

-Absorption takes place through simple diffusion and active transport

-High intake reduces absorption [70]

FUNCTIONAL ROLE [68,69]

1. ANTIOXIDANT

It serves as an antioxidant due to its ability to react with free radicals and undergo a single-electron oxidation process to form ascorbyl radical, a poorly reactive intermediate which disproportionates to ascorbate and dehydroascorbate.[69]

- First line of defense against ROS in plasma, interstitial fluids and soluble phases of cells.

- Protects Lipid, DNA and Nitric oxide from oxidation.
- Regenerates the metabolically active (reduced) form of Vitamin E (tocopherol form tocopheroxyl radical) – **synergistic action**
- Protects Glutathione in its reduced form by quenching of oxidants.

IN DIABETES

- Reduces insulin resistance by inhibiting oxidant induced serine phosphorylation of IRS and enhancing tyrosine kinase activity, thereby improving downstream insulin signal transduction.
- Regenerates other antioxidants, helps in maintaining antioxidant pool.
- Reduces glycosylation of plasma proteins suggesting a role in preventing diabetic complications[71]
- Reduces sorbitol accumulation within erythrocytes by inhibiting aldose reductase activity.[72]

2. ENZYMATIC CO-SUBSTRATE FUNCIONS

- Helps in synthesis of collagen by hydroxylation of prolyl and lysyl residues.[73]
- Catalyses ferrochelatase and incorporates iron into protoporphyrin IX to form heme
- Acts as co-factor in synthesis of carnitine

- Serves as an electron donor in hydroxylation reaction of dopamine to form norepinephrine.[73]

3. CARDIOVASCULAR HEALTH

- Anti-atherogenic effect by reducing oxidation of LDL.
- Improves exercise tolerance by its effect on reducing endothelial dysfunction [74]

  (Protects nitric oxide (NO), a major vasodilator from oxidation).

4. EFFECT ON BLOOD PRESSURE

- Protecting NO from inactivation by ROS and promoting vasodilatation.
- Improving arterial stiffness by protecting cell membrane pumps from oxidative damage and promoting ion flux.
- Modulating the autonomic nervous system by restoring sympathovagal balance and improving spontaneous baroreceptor sensitivity.[75]
- These effects result in lowering of blood pressure.

5. PROMOTION OF IRON BIOAVAILABILITY

- Increases iron availability in foods due to increased enteric absorption.

- Also promotes utilization of heme iron by enhanced incorporation into Ferritin.

6. METABOLISM OF CHOLESTEROL

- Cholesterol is routinely converted into bile acids in the body. For this transformation reaction, hydroxyl group is incorporated into cholesterol nucleus with the help of vitamin C.
- By increasing the expression of LDL receptor in the liver it helps in LDL degradation.[76]

These mechanisms help reduces serum cholesterol levels.

7. IMMUNITY

- It modulates Phagocytic activity, Lymphocytes and cytokine production, Interferon production,T cell gene expression and synthesis of IgG and IgM antibodies [69]

8. ANTI INFLAMMATORY ACTION

- Supresses the production of 8isoPGF2α, the first identified isoprostane produced by non-enzymatic free radical induced lipid peroxidation.
- Reduces biomarkers of inflammation (c-reactive protein) and endothelial dysfunction (tissue plasminogen activator)

9. CATARACT

- Protects lens from photo-oxidative effects of Ultraviolet light.
- Prevents development of cataract by reducing sorbitol accumulation in the lens.

10. CANCER

- Vitamin C is a potent inhibitor of nitrosamine-induced carcinogenesis, by functioning as a nitrite scavenger.[69]

11. MISCELLANEOUS

- Improves Bone health * Promotes wound healing

DEFICIENCY [69]

-Scurvy -Weak bones

-Bleeding tendencies - Delayed wound healing

-Teeth malformation - Decreased iron content of body

- Painful joints - Malaise - Depression

ADVERSE EFFECTS

(High dose >2g/day)

- Mild GI upset
- Headache
- Sleep disturbances
- Oxalate stones in kidney (dose >4g/day) [77]

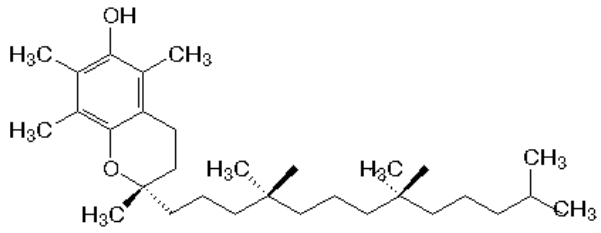
USES [77]

- Scurvy – prevention and treatment
- Anemia
- Non healing leg ulcer
- Bed sores
- Common cold
- Methaemoglobinemia- as a reducing agent.
- To acidify urine in urinary tract infections [78]

VITAMIN E

- Fat soluble vitamin
- Vitamin E available as eight forms: Tocopherol -4 ,tocotrienols -4
- Most common active form is α-tocopherol

CHEMICAL STRUCTURE



Vitamin E (α-tocopherol)

Has a chromanol ring and C-6 hydroxyl group (which donates hydrogen for reducing free radicals) and hydrophobic side chain (for penetrating biological membranes)[79]

SOURCES

-Wheat germ oil	- Rice bran oil	-Soyabean oil	- Nuts
- Sunflower oil	- Safflower oil	- Fish	- Egg yolk
- Cereals	- Spinach	-Green leafy vegetables [69]	

PHARMACOKINETIC PARAMETERS

- Primarily absorbed in medial small intestine
- Does not have a specific carrier in plasma and is rapidly transported from chylomicrons to plasma lipoproteins or directly to tissues
- Metabolized in liver by oxidation (CYP enzymes) and excreted in urine and bile
- In non-adipose cells, vitamin E is localized almost exclusively in membranes.[80]

FUNCTIONAL ROLE

1. ANTI OXIDANT

Vitamin E is the major antioxidant in membranes and has **membrane stabilizing action.** It gets incorporated into the phospholipid bilayer and protects the PUFAs of the cellular membranes from free radical induced oxidative damage.[81] In serving its antioxidant function, it gets converted to tocopheroxyl radical which is unreactive and thus it stops the destructive propagative cycle of oxidative damage. It is regenerated to its active form by Vitamin C.

IN DIABETES

- In Diabetes due to oxidative stress there is increased lipid peroxidation, particularly in erythrocytes which leads to their premature lysis. Vitamin E (**synergestic with Vitamin C**) prevents oxidation of PUFAs in the cell membrane, thereby maintaining integrity of the RBC membrane, reducing fragility and oxidative stress induced hemolysis.
- Vitamin E may have a role as an erythropoietic factor, thus improving anemia[82]
- Lowers urinary and serum F2 isoprostanes levels in diabetics, an important marker of oxidative stress.[83]
- Reduces insulin resistance by increasing tyrosine kinase activity.

2. IMMUNITY

- Modulates T cell function, stimulates production of lymphocytes and antibodies. Promotes Phagocytosis.[81]

3. ANTIINFLAMMATORY ACTION

- Inhibits Phospholipase A2 activity, thus preventing release of Arachidonic acid and subsequent generation of Prostaglandins and Leukotrienes.
- Inhibit release of proinflammatory cytokines from activated macrophages.[81]

4. CARDIOVASCULAR HEALTH

- Prevents LDL oxidation and subsequent atherosclerotic plaque formation.[84]
- Reduces platelet aggregation[85]

5. ANALGESIC ACTION

- Reduces central pain processing by suppression of nitric oxide (NO lowers the threshold of nociceptors thus facilitating pain transmission pathway) [86]

6. ANTICARCINOGENESIS

- Selectively stimulate apoptosis in certain neoplastic cells by receptor induced caspase -8 and -3 activation in some cancer cells and caspase -9 activation in others.[84]

7. CATARACTS

- Direct antioxidant effect or indirect antioxidant effect in maintaining lens glutathione in reduced state, it has shown to lower risk of cataract formation.[81]

DEFICIENCY SYMPTOMS:[81]

*Loss of appetite

*Peripheral neuropathy

*Retinopathy

*Myopathies [87]

*Spinocerebellar Ataxia

*Decreased immunity

*RBC hemolysis, anemia

ADVERSE DRUG REACTIONS

It is viewed as one of the least toxic vitamins with doses upto 400 IU/day tolerated well.

High dose (>2000 mg/day) can produce:

- Headache - Double vision
- Muscle weakness - GI upset, nausea - Creatinuria
- Bleeding tendency: due to reduced Vitamin K – dependant carboxylase activity. [88]

USES [88]

- Diabetes mellitus
- Haemolytic anemia of prematurity
- Chronic hemolysis in G6PD deficiency
- Acanthocytosis
- Intermittent claudication
- Benign breast tumour
- Nocturnal leg cramps
- Sterility
- Atherosclerosis
- Parkinson's disease
- Alzeihmer's disease.

AIM AND

OBJECTIVES

AIM AND OBJECTIVE

AIM:

To study the efficacy of Vitamin E and Vitamin C in Type 2 Diabetes Mellitus.

OBJECTIVES:

PRIMARY OBJECTIVE:

To study red blood cell morphology in early Type 2 Diabetes patients as a marker of oxidative stress and the efficacy of Antioxidants such as Vitamin C and E as an add on therapy to standard treatment.

SECONDARY OBJECTIVE:

Improvement in glycemic control and haemoglobin level.

METHODOLOGY

METHODOLOGY

STUDY DESIGN:

Randomized, Open label, Comparative Pilot study

STUDY POPULATION:

Adult patients with early Type 2 Diabetes Mellitus attending the diabetic outpatient department.

STUDY CENTER:

Institute of Diabetology, Madras medical college & Rajiv Gandhi Government General Hospital, Chennai.

STUDY PERIOD:

January 2015 – December 2015

SAMPLE SIZE:

60 patients (30 patients in control group and 30 patients in study group)

STUDY DURATION:

8 weeks study period and 4 weeks follow up per patient

ELIGIBILITY CRITERIA

INCLUSION CRITERIA:

- Age: 30-70 years
- Sex-both genders
- Patients diagnosed with Type 2 Diabetes Mellitus within 2 yrs
- Patients on metformin monotherapy.
- Patients willing to give written informed consent

EXCLUSION CRITERIA:

- Patients with Type 1 Diabetes Mellitus
- Current or ex smokers
- Pregnant and lactating women
- Patients with co-existing liver disease, heart disease, renal disease or malignancy
- Patients on any lipid lowering drugs
- Patients with any diagnosed hematological disorder
- Patient enrolled in any other study

STUDY PROCEDURE

The study was conducted after obtaining approval from the Institutional Ethics Committee, Madras Medical College and was done in accordance with Declaration of Helsinki and Good Clinical Practice (GCP) guidelines. Patients diagnosed with Type 2 Diabetes Mellitus within the last 2 years on metformin monotherapy were recruited from the outpatient department of the Institute of Diabetology, Madras Medical College & Rajiv Gandhi Government General Hospital. The purpose procedures and benefits of the study were explained to them. Written informed consent was obtained from the subjects who were willing to participate in the trial in the prescribed format in regional language prior to performance of any study related procedure. If the patient was illiterate, left thumb impression in the presence of an impartial witness was taken.

The demographic details of the patients was obtained and recorded. Patients were screened by History, General & Systemic examinations and Lab investigations. Patients who fulfilled the inclusion and exclusion criteria were enrolled and randomized to either the study group or control group.

The following **lab investigations** were performed during screening.

- Fasting blood sugar
- Haemoglobin
- Total Red blood cell count
- Liver function tests
- Renal function tests

RECRUITMENT:

- 102 patients were screened and 30 patients in each group (control and study groups) who fulfilled the inclusion and exclusion criteria were recruited into the study.

- No drop-out of patients occurred in either of the groups

RANDOMIZATION

The enrolled patients were randomized by simple randomization into control and study group and received the respective therapies.

TREATMENT PLAN

GROUP A - Control group(n=30): patients received standard treatment.

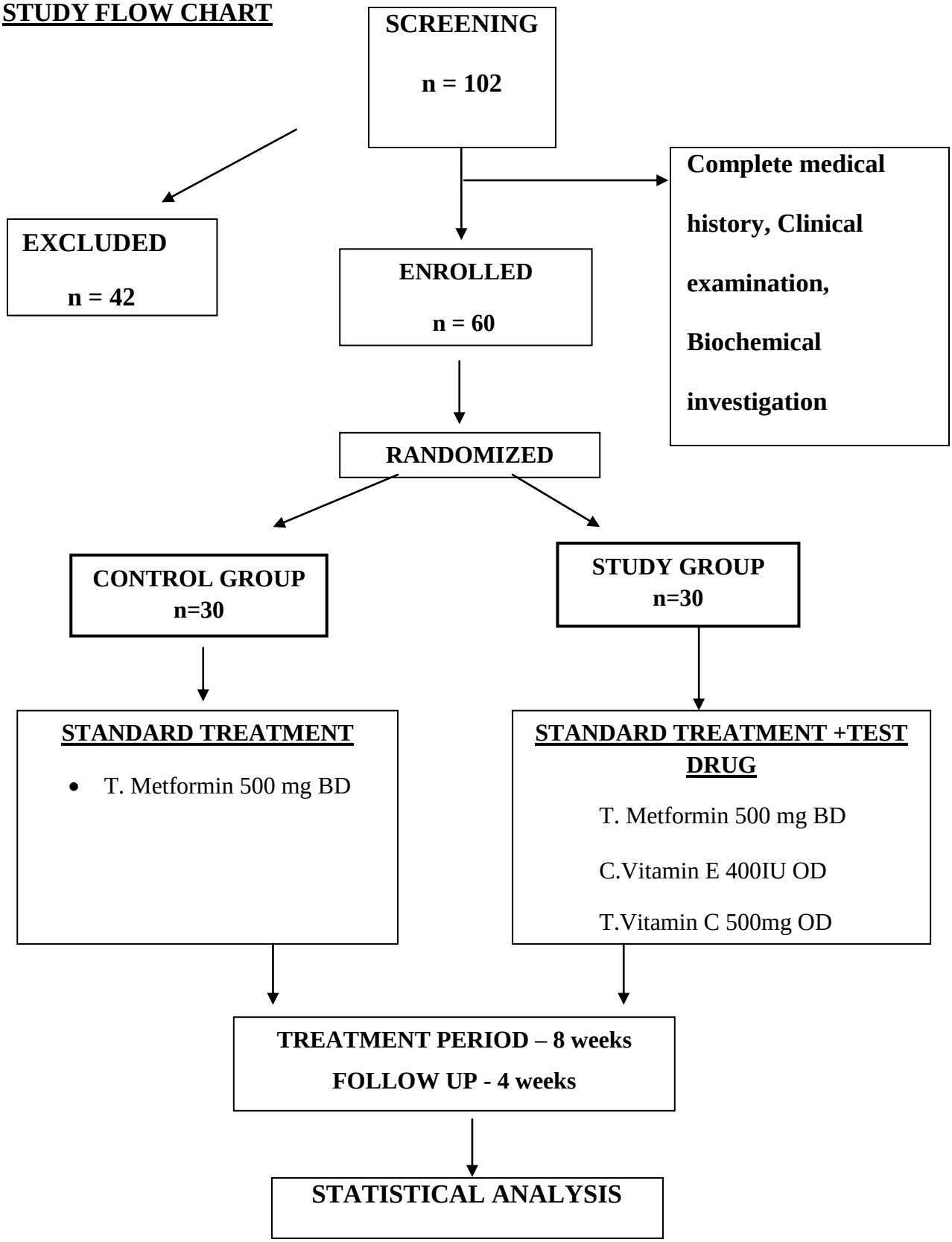
Standard treatment:

- Tablet Metformin 500 mg twice daily

GROUP B - Study group (n=30): patients received standard treatment plus one tablet of Vitamin C -500mg and one capsule of Vitamin E -400IU each once daily.

The patients received the study medication for 4 weeks at a time and were asked to review at the end of 4 weeks and 8 weeks with the empty blister pack to check compliance.

STUDY FLOW CHART



SCHEDULED VISITS

SCREENING:

- Written Informed consent obtained
- Demographic details obtained
- Medical history obtained
- General & systemic examination done
- Laboratory investigations done
- Enrolment done.

Visit 1

- Randomization done
- Vital signs recorded
- RBC morphology studied
- Fasting blood sugar, haemoglobin and RBC count measured
- Study medications issued for 4 weeks
- Instructed to return empty strips during subsequent visit
- Asked to report any adverse event, if any occurs.

Visit 2 (end of 4 weeks)

- Vital signs recorded
- Asked for return of empty strips to assess compliance
- Adverse events recorded, if any
- RBC morphology studied
- Fasting blood sugar, haemoglobin and RBC count measured
- Study medications issued for another 4 weeks

Visit 3 (end of 8 weeks)

- Vital signs recorded.
- RBC morphology studied
- Fasting blood sugar, haemoglobin and RBC count measured
- Adverse events recorded, if any

Visit 4 (end of 12 weeks)

- Vital signs recorded
- Adverse events recorded, if any
- RBC morphology studied
- Fasting blood sugar, haemoglobin and RBC count measured

ASSESSMENT

I**. Morphological changes in RBCs**: 1ml of the patient's blood was centrifuged at 3000 rpm for 5 minutes. The supernatant was discarded and the packed cells diluted with equal volume of 0.9% normal saline and centrifuged again. The supernatant was again discarded and the packed cells reconstituted as 10% v/v suspension with 0.9% normal saline and a drop of this suspension was put on a glass slide under a cover slip and studied under high power microscope.

Under the high power microscope, a central field was selected. 100 RBC's were veiwed and the number of Crenated RBCs with Heinz bodies were counted for each patient in both the groups at each visit and recorded as a percentage.

II. **Glycemic control-** based on fasting blood sugar levels

III. **Hemoglobin levels and RBC count –** was recorded at each visit

Differences in the counts before and after treatment in the both the groups were recorded.

Lab investigations:

The following additional investigations were repeated for the patients at the end of 8 weeks of treatment.

- Blood urea
- Serum creatinine
- Liver function tests

INSTRUCTION TO PATIENTS

The patients were instructed clearly regarding the regular intake of the medicines. They were given proper advice to report for assessment and drug receiving. They were counseled to report if any acute complaints, reactions occur.

FOLLOW UP

The patients were followed up for a post treatment period of 4 weeks, without the study drug for the assessment of vitals, Hemoglobin, RBC count and RBC morphology.

After the completion of 12 weeks of study period, the patients were provided appropriate medical care at Institute of Diabetology, Rajiv Gandhi Government General Hospital, Chennai.

ADVERSE EVENTS

Any adverse event reported by the patient or observed by the physician during the study was recorded. The onset of adverse event, causal relationship to the study drug and action taken was recorded. Appropriate medical care was provided.

WITHDRAWAL

During the study period the subject was allowed to withdraw his/her voluntary consent and opt out of study. Similarly at the discretion of the investigator, the subjects were withdrawn from the study if any serious adverse event reported by the patient or observed by the physician.

STATISTICAL ANALYSIS

The obtained data was analyzed statistically.

Distribution of age was analyzed using one way ANOVA and Sex distribution was analyzed by Pearson chi- square test.

The difference within the groups in hemoglobin level, RBC count, RBC morphology, Systolic and Diastolic blood pressure was analyzed using students paired t-test. Similarly the difference between the control and test groups was analyzed using independent t-test.

The biochemical investigations were performed at baseline and at the end of 8 weeks. The differences within the groups before and after treatment were analyzed using student's paired t- test.

Statistical analysis was done using SPSS vs 20.

P value <0.05 was considered to be statistically significant.

RESULTS

TABLE 1: MEAN AGE DISTRIBUTION

GROUPS	n (No. of patients)	MEAN AGE (in years)	SD	p VALUE
CONTROL	30	48.33	9.19	**0.844**
STUDY	30	47.87	9.06	

Table 1 – shows the mean age distribution of patients among control and study groups.

The mean age of patients in control group is 48.33 and in the study group is 47.87 (p = 0.844), indicating that there is no significant difference in age between the two groups.

FIGURE 1: MEAN AGE DISTRIBUTION

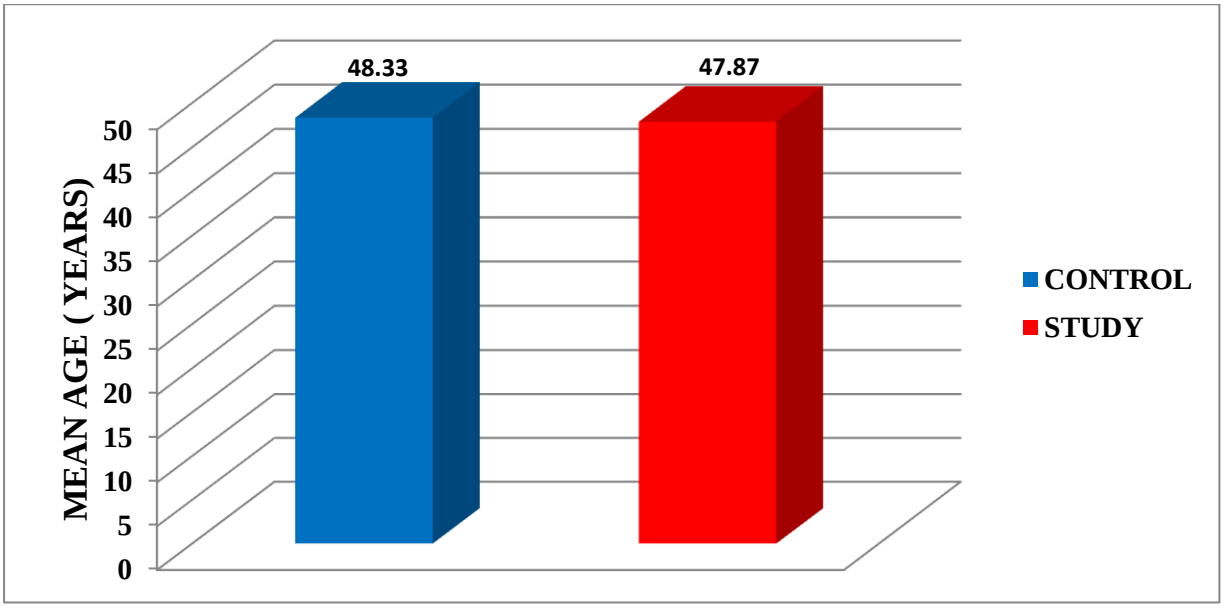


Fig 1 is a graphical representation of Table 1.

TABLE 2: GENDER DISTRIBUTION

SEX DISTRIBUTION	GROUPS			
	CONTROL		STUDY	
	n	%	n	%
MALE	10	33%	11	37%
FEMALE	20	67%	19	63%
TOTAL NO. OF PATIENTS	30	100%	30	100%

Table 2 - shows the distribution of male and female patients in the two groups

In control group, 10 patients are male (33%) and 20 patients are female (67%) and in study group, 11 patients are male (37%) and 19 patients are female (63%). There is no significant difference in sex distribution.

FIGURE 2: GENDER DISTRIBUTION

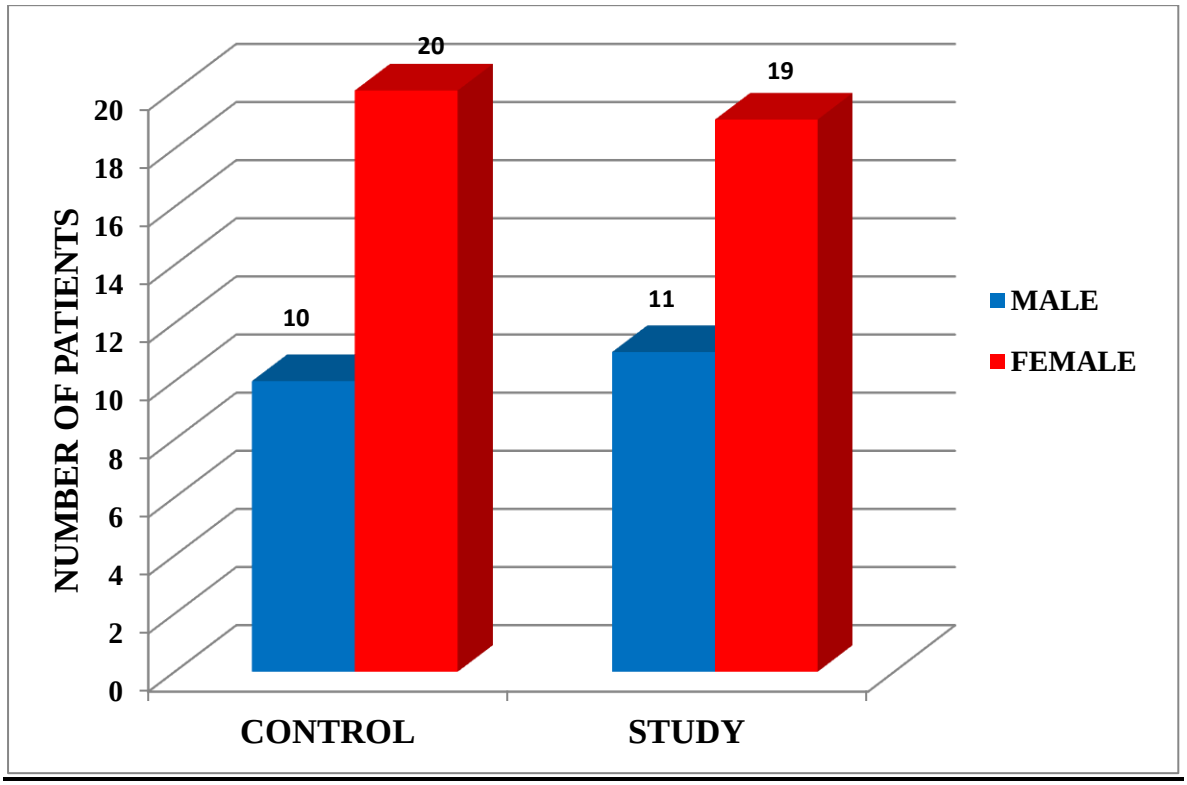


Fig 2 is a graphical representation of Table 2

TABLE 3: MEAN DURATION OF DIABETES (MONTHS)

GROUPS	n (NO. OF PATIENTS)	MEAN DURATION (MONTHS)	SD	p VALUE
CONTROL	30	11.4	6.7	0.175
STUDY	30	9.13	5.9	

Table 3 – shows the mean duration of diabetes among the patients in the control and study group.

The mean duration of diabetes in patients in the control group is 11.4 months and in the study group is 9.13 months. p = 0.175 indicating that there is no significant difference in the mean duration of diabetes between the two groups.

FIGURE 3: MEAN DURATION OF DIABETES (MONTHS)

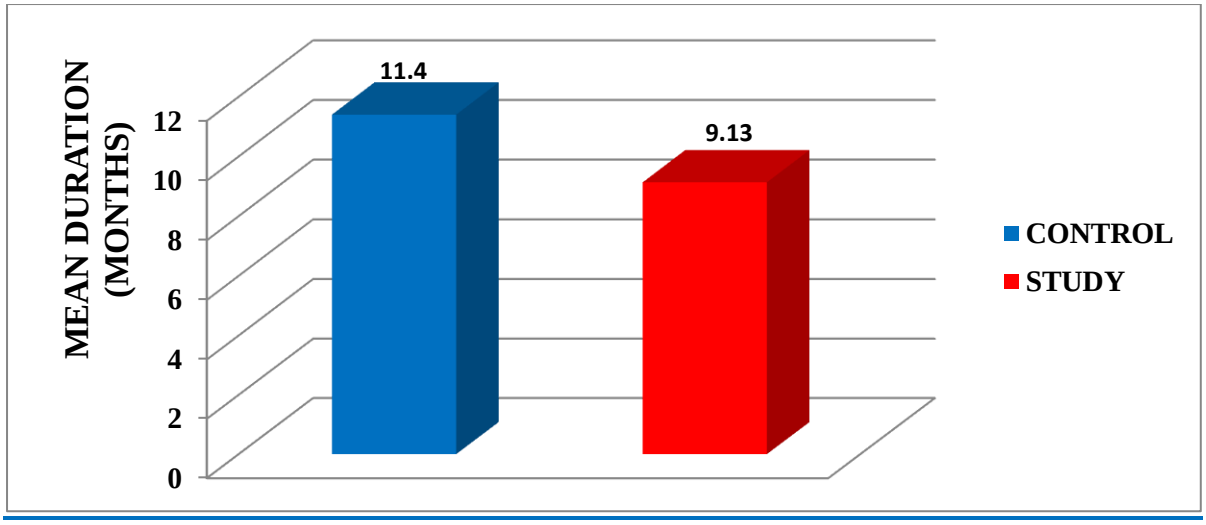


Fig 3 is a graphical representation of Table 3

TABLE 4 : MEAN FASTING BLOOD GLUCOSE (mg/dl)

GROUPS	DAY 0		AT THE END OF 8 WEEKS		p value
	MEAN (mg/dl)	SD	MEAN (mg/dl)	SD	
CONTROL	185.2	57.66	144.8	29.58	0.030
STUDY	181.6	27.98	117.3	15.77	0.001
p value	0.762		0.001		

Table 4- shows the mean fasting blood glucose levels in control and study groups.

On comparing the two groups,

There is a statistically significant reduction within the control group (p = 0.030) and within the study group (p=0.001) in fasting blood glucose levels at the end of 8 weeks.

The difference between the control and study groups on day 0 (p = 0.762) is insignificant but and at the end of 8 weeks (p=0.001) there is a statistically significant reduction in fasting blood glucose level in study group compared to the control group.

FIGURE 4 : MEAN FASTING BLOOD GLUCOSE (mg/dl)

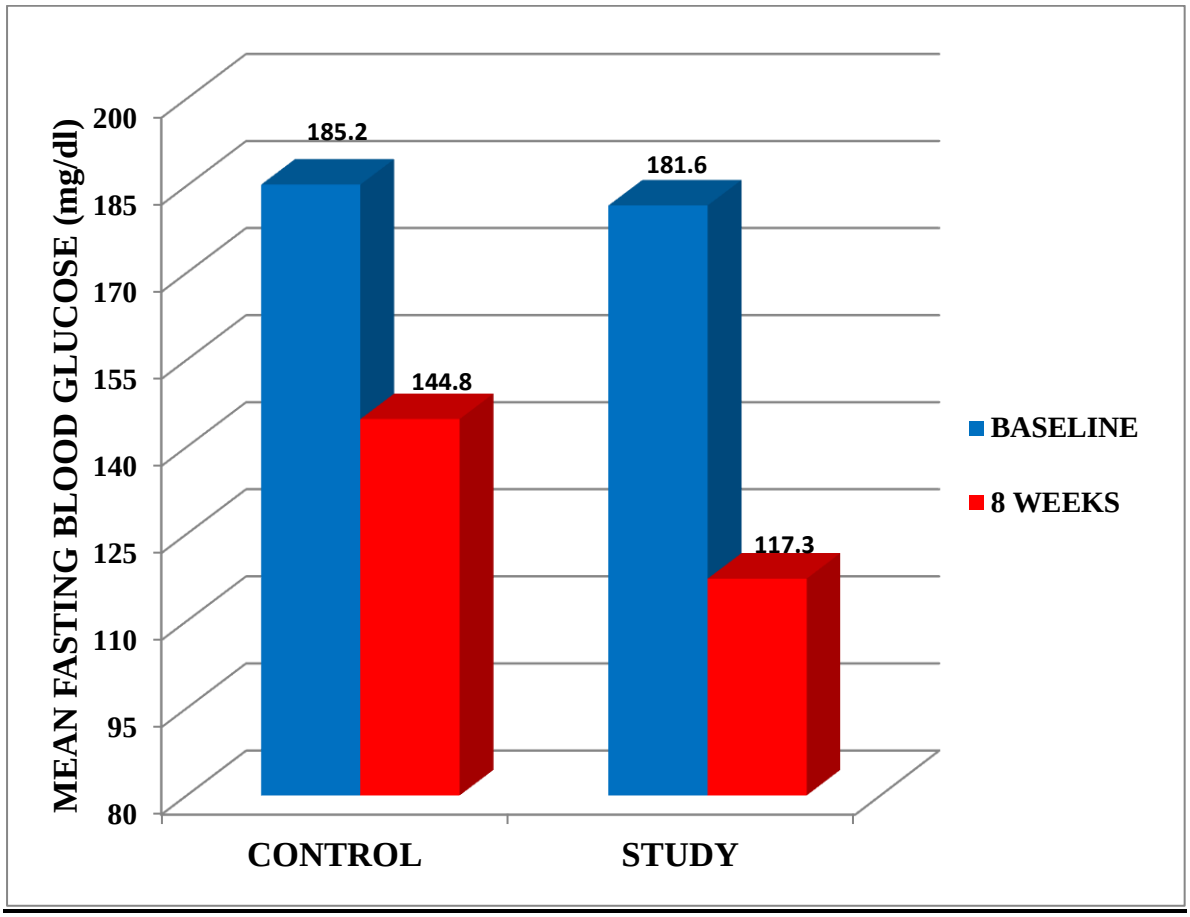


Fig 4 is a graphical representation of Table 4

TABLE 5 – MEAN PERCENTAGE OF CRENATED RBCs WITH HEINZ BODIES (%)

GROUPS	DAY 0		AT THE END OF 8 WEEKS		p value
	MEAN (%)	SD	MEAN (%)	SD	
CONTROL	80.83	13.06	76.87	12.79	0.051
STUDY	84.67	12.82	6.93	2.16	0.001
p value	0.256		0.001		

Table 5 - shows the mean percentage of crenated red blood cells with Heinz bodies in the control and study groups

On comparing the two groups,

There is no statistically significant change within the control group (p =0.051) but the study group shows significant reduction in the % of crenated RBCs at the end of 8 weeks (p=0.001).

There is no significant difference between the control and study group on day 0 (p = 0.256) But at the end of 8 weeks study group shows a significant reduction in the % of crenated RBCs compared to control group (p=0.001) .

FIGURE 5: MEAN PERCENTAGE OF CRENATED RBCs WITH HEINZ BODIES (%)

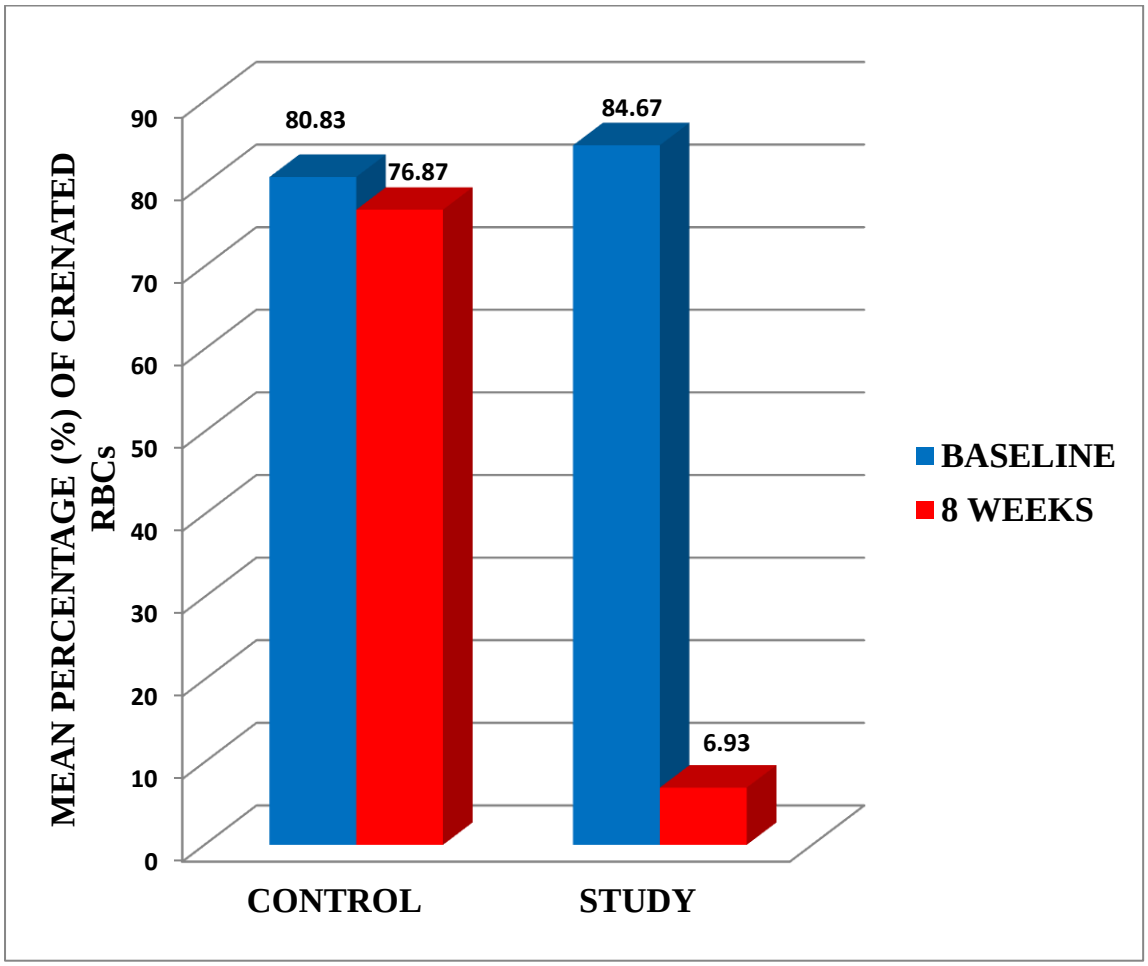
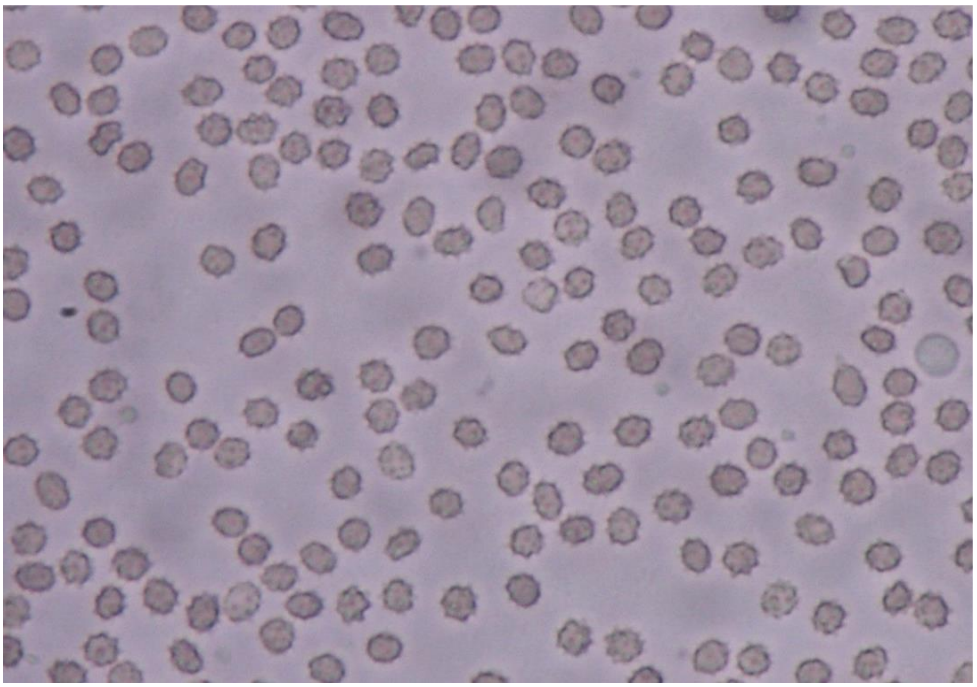
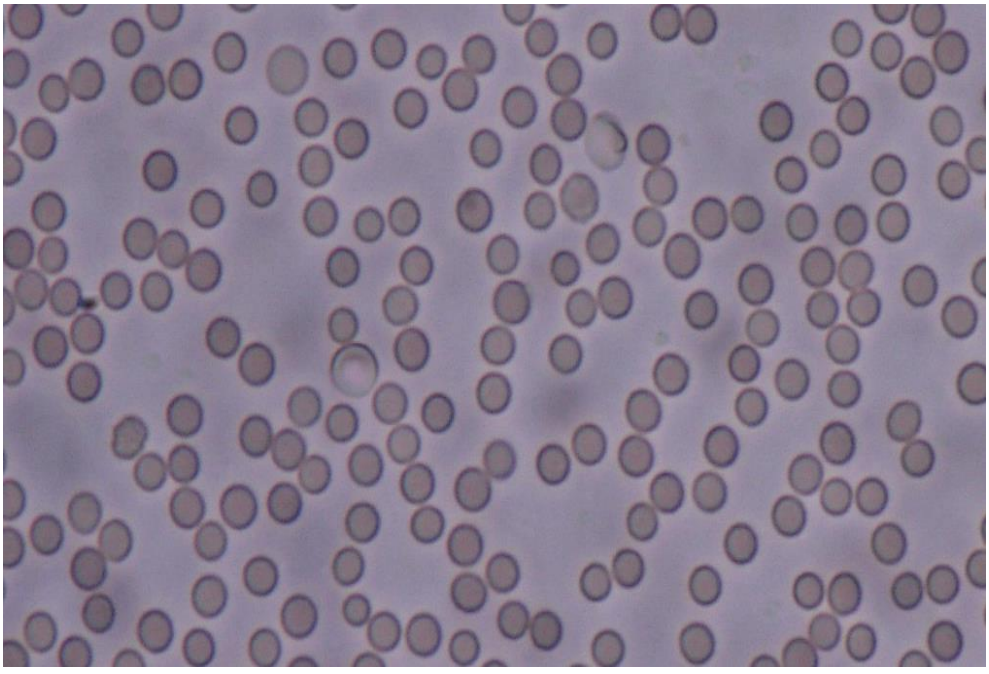


Fig 5 is a graphical representation of Table 5.

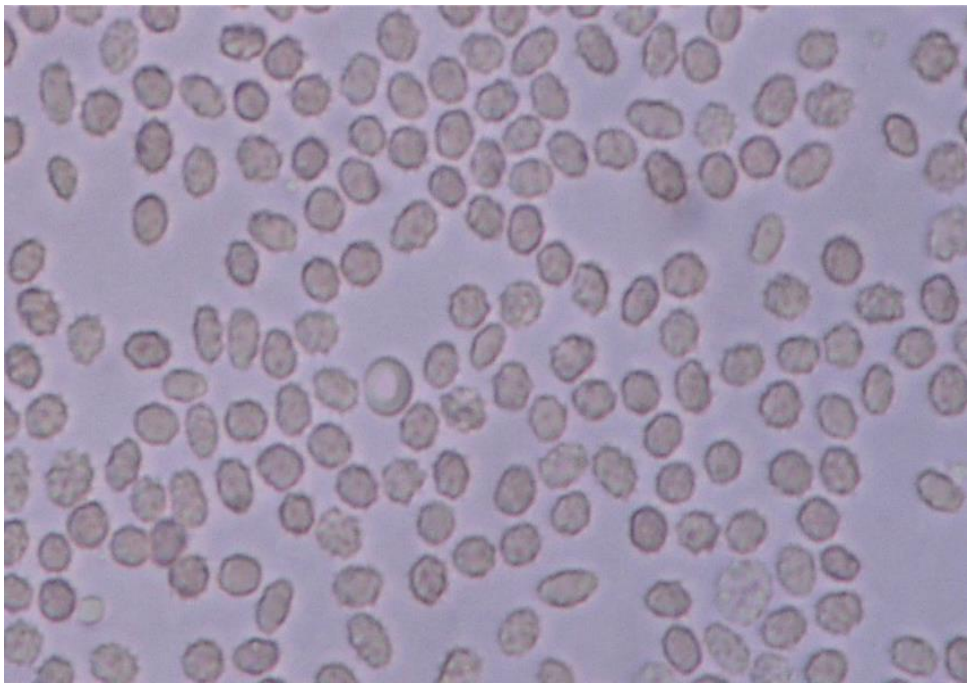
MORPHOLOGY OF RBCs BEFORE TREATMENT



MORPHOLOGY OF RBCs AFTER TREATMENT



MORPHOLOGY OF RBCs BEFORE TREATMENT



MORPHOLOGY OF RBCs AFTER TREATMENT

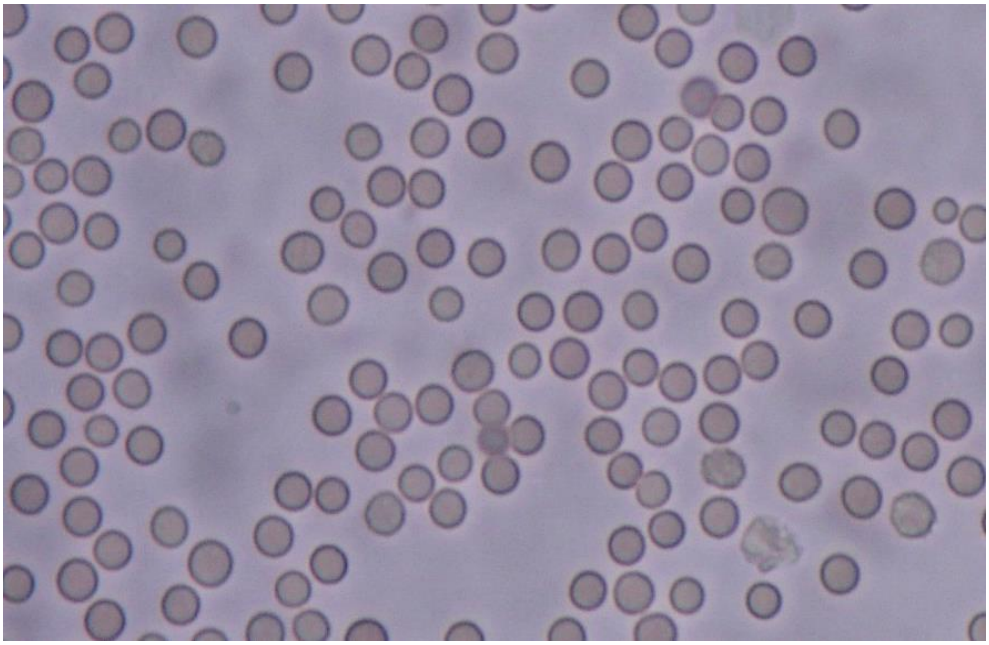


TABLE 6A: MEAN HAEMOGLOBIN(gm%)

GROUPS	DAY 0		AT THE END OF 8 WEEKS		p value
	MEAN (gm%)	SD	MEAN (gm%)	SD	
CONTROL	10.367	1.311	10.343	1.30	0.690
STUDY	10.190	1.534	11.450	1.462	0.001
p value	0.633		0.003		

Table 6A - shows the mean hemoglobin values of patients in the control and study group. On comparing the two groups,

There is no statistically significant change within the control group (p =0.690) but the study group shows significant increase in mean haemoglobin at the end of 8 weeks (p=0.001).

There is no significant difference between the control and study group on day0 (p = 0.633). But at the end of 8 weeks, the study group shows a significant increase in mean haemoglobin compared to the control group (p=0.003).

FIGURE 6A: MEAN HAEMOGLOBIN(gm%)

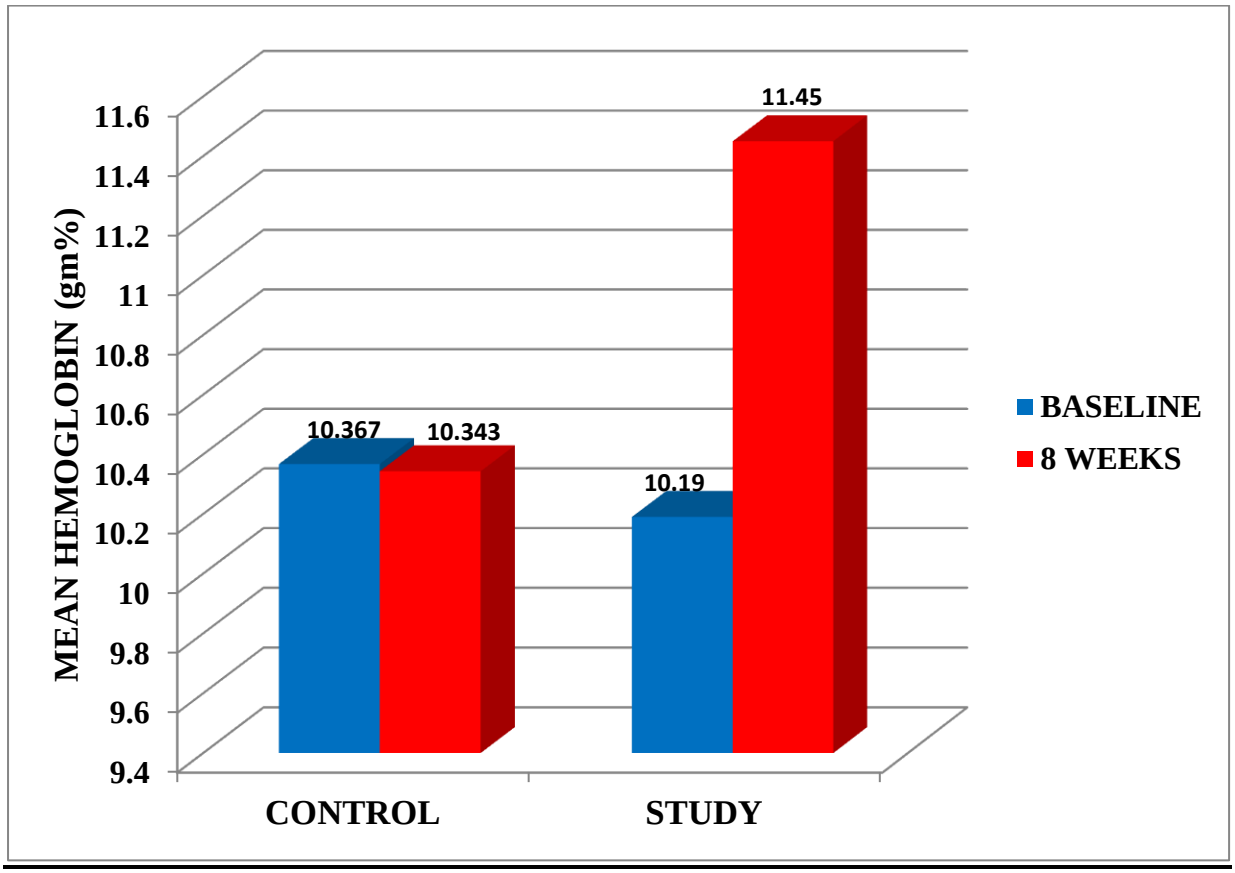


Fig 6A is a graphical representation of Table 6A

TABLE 6B – NUMBER (%) OF PATIENTS WITH ANEMIA

HEMOGLOBIN (g/%)	CONTROL (n=30)	STUDY (n = 30)
<12 gm% (ANEMIC)	24 (80%)	25 (83%)
≥12 gm%(NOT ANEMIC)	6 (20%)	5 (17%)

Table 6B – shows the number (%) of patients with anemia (Hb <12gm%).

Majority of the patients in the control group(80%) and study group(83%) had anemia with hemoglobin of less than 12 gm%.

FIGURE 6B: NUMBER OF PATIENTS WITH ANEMIA

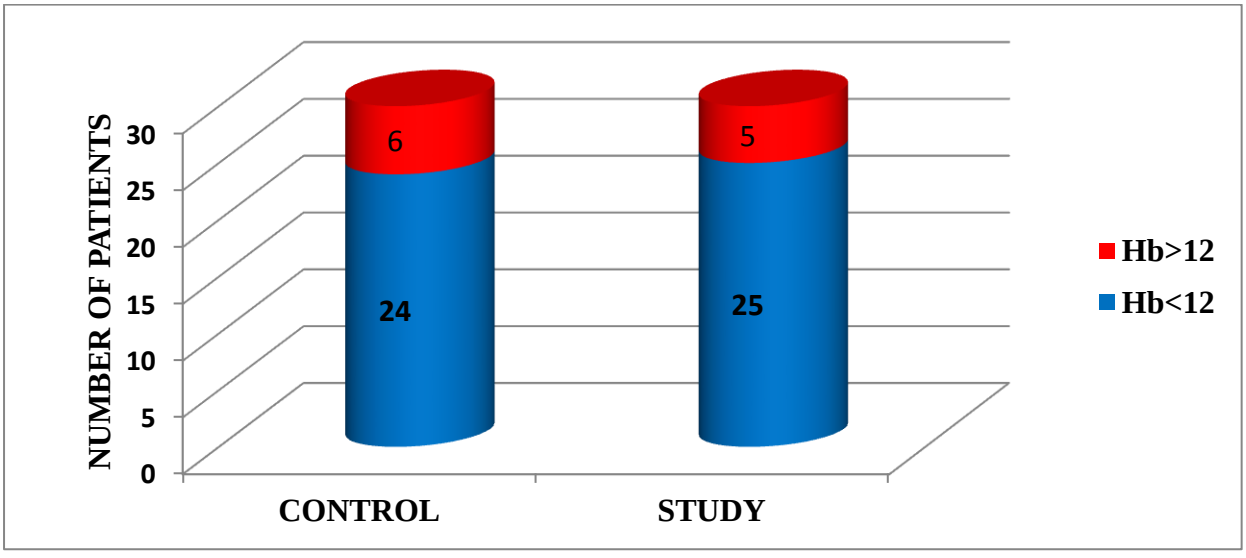


Figure 6B is a graphical representation of Table 6B

TABLE 7: MEAN RED BLOOD CELL(RBC) COUNT(millions/µL)

GROUPS	DAY 0		AT THE END OF 8 WEEKS		p value
	MEAN (millions/µL)	SD	MEAN (millions/µL)	SD	
CONTROL	3.523	0.454	3.512	0.451	0.624
STUDY	3.48	0.514	3.886	0.489	0.001
p value	0.730		0.003		

Table 7 - shows the mean Red blood cell (RBC) count in patients of the control and study groups

On comparing the two groups,

There is no statistically significant change within the control group

(p =0.624) but the study group shows a significant increase in RBC count at the end of 8 weeks (p=0.001).

There is no significant difference between the control and study groups on day 0 (p = 0.730). But at the end of 8 weeks the study group shows a significant increase in the RBC count compared to the control group (p=0.003).

FIGURE 7: MEAN RED BLOOD CELL COUNT(millions/µL)

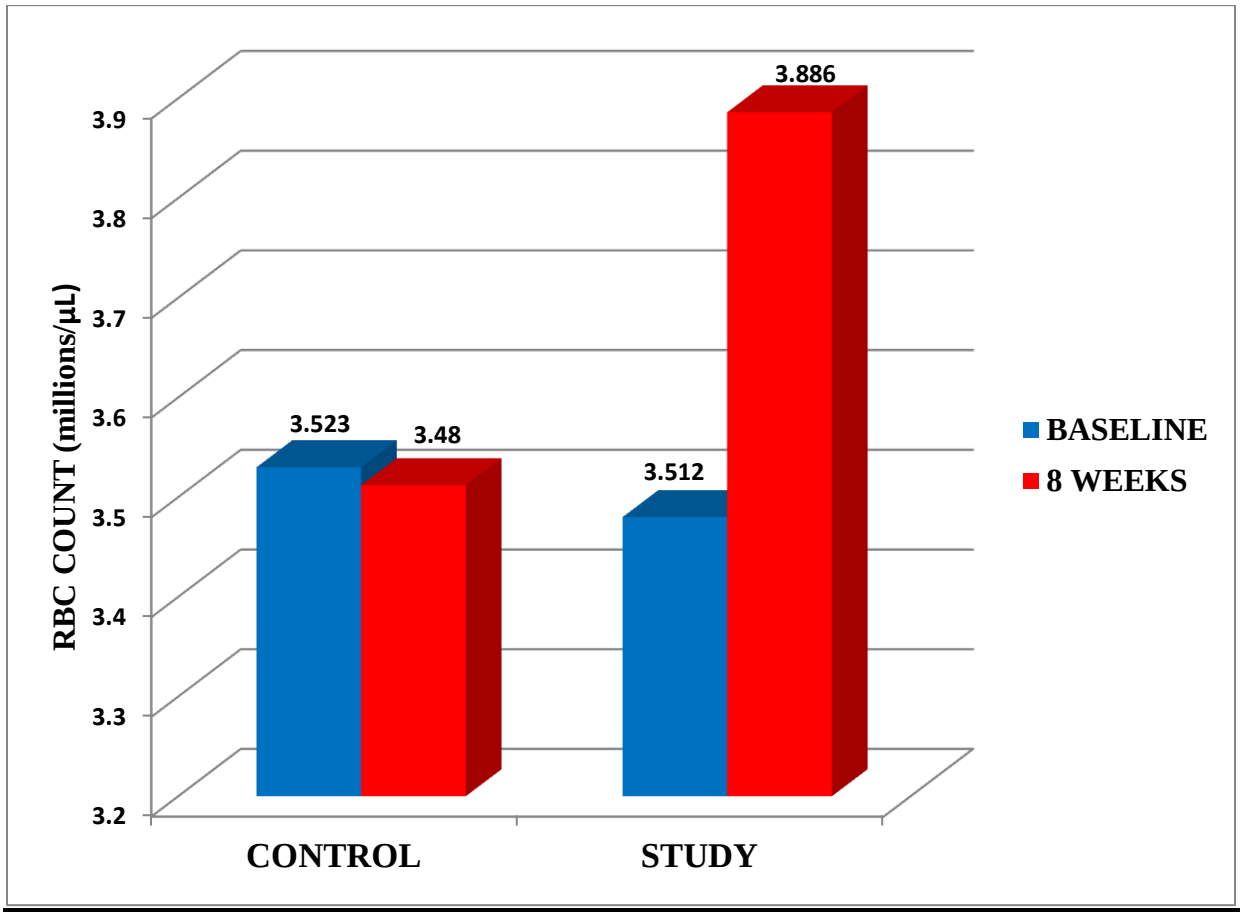


Fig 7 is a graphical representation of Table 7

TABLE 8 : MEAN SYSTOLIC BLOOD PRESSURE(mmHg)

GROUPS	DAY 0		AT THE END OF 8 WEEKS		p value
	MEAN (mm Hg)	SD	MEAN (mm Hg)	SD	
CONTROL	124.2	10.691	126.3	9.681	0.436
STUDY	126.8	9.792	120.6	11.34	0.027
p value	0.330		0.042		

Table 8 - shows the mean systolic blood pressure in control and study groups

On comparing the two groups,

There is no statistically significant change within the control group

(p =0.436) but the study group shows significant reduction in mean systolic blood pressure at the end of 8 weeks (p=0.027).

There is no significant difference between the control and study group on day 0 (p = 0.330) But at the end of 8 weeks there is significant reduction in systolic blood pressure in study group compared to control group (p=0.042).

FIGURE 8: MEAN SYSTOLIC BLOOD PRESSURE (mmHg)

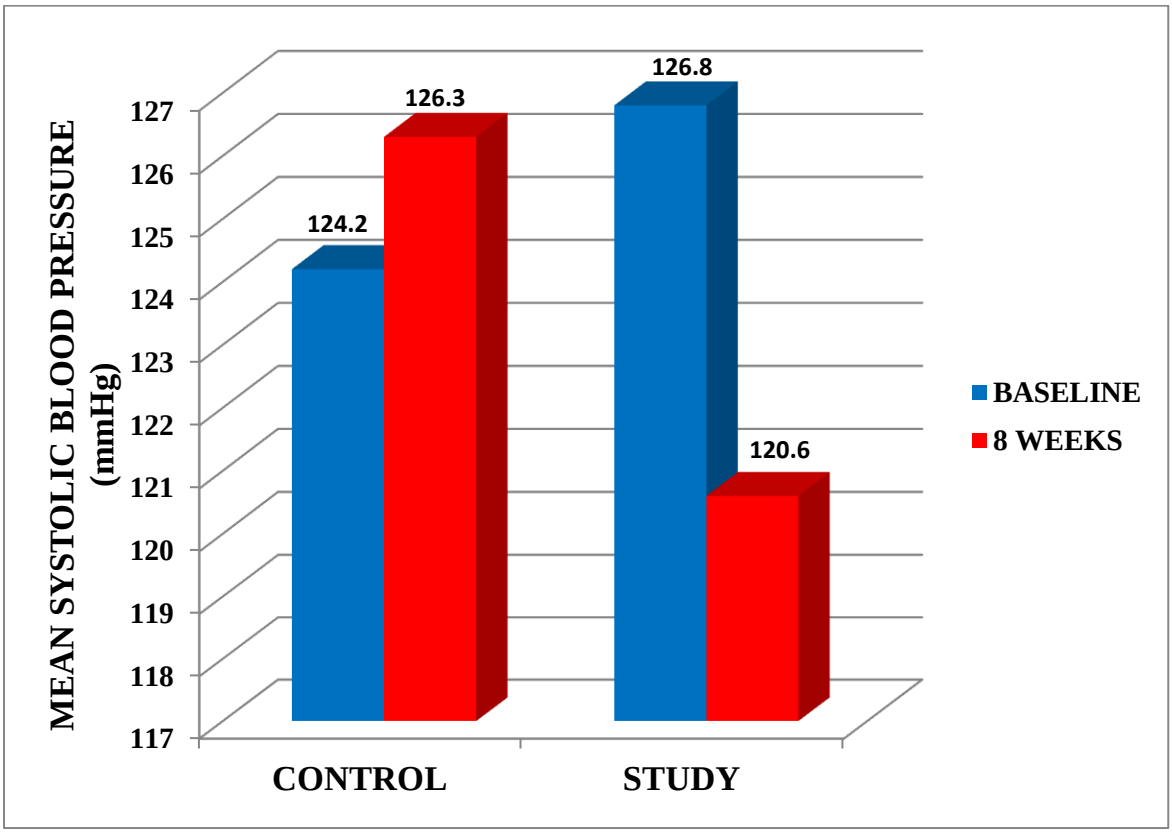


Fig 8 is a graphical representation of Table 8

TABLE 9 : MEAN DIASTOLIC BLOOD PRESSURE (mmHg)

GROUPS	DAY 0		AT THE END OF 8 WEEKS		p value
	MEAN BP (mm Hg)	SD	MEAN BP (mm Hg)	SD	
CONTROL	80.6	6.790	80.4	6.526	0.908
STUDY	81.4	7.596	76.4	7.762	0.014
p value	0.669		0.035		

Table 9 - shows the mean diastolic blood pressure in control and study groups.

On comparing the two groups,

There is no statistically significant change within the control group

(p =0.908). However there is significant reduction in the mean diastolic blood pressure in the study group (p=0.014).

The difference between the control and study groups on day 0 (p =0.669) is not significant but there is significant difference in the reduction of diastolic blood pressure at the end of 8 weeks between control and study groups (p=0.035).

FIGURE 9: MEAN DIASTOLIC BLOOD PRESSURE(mmHg)

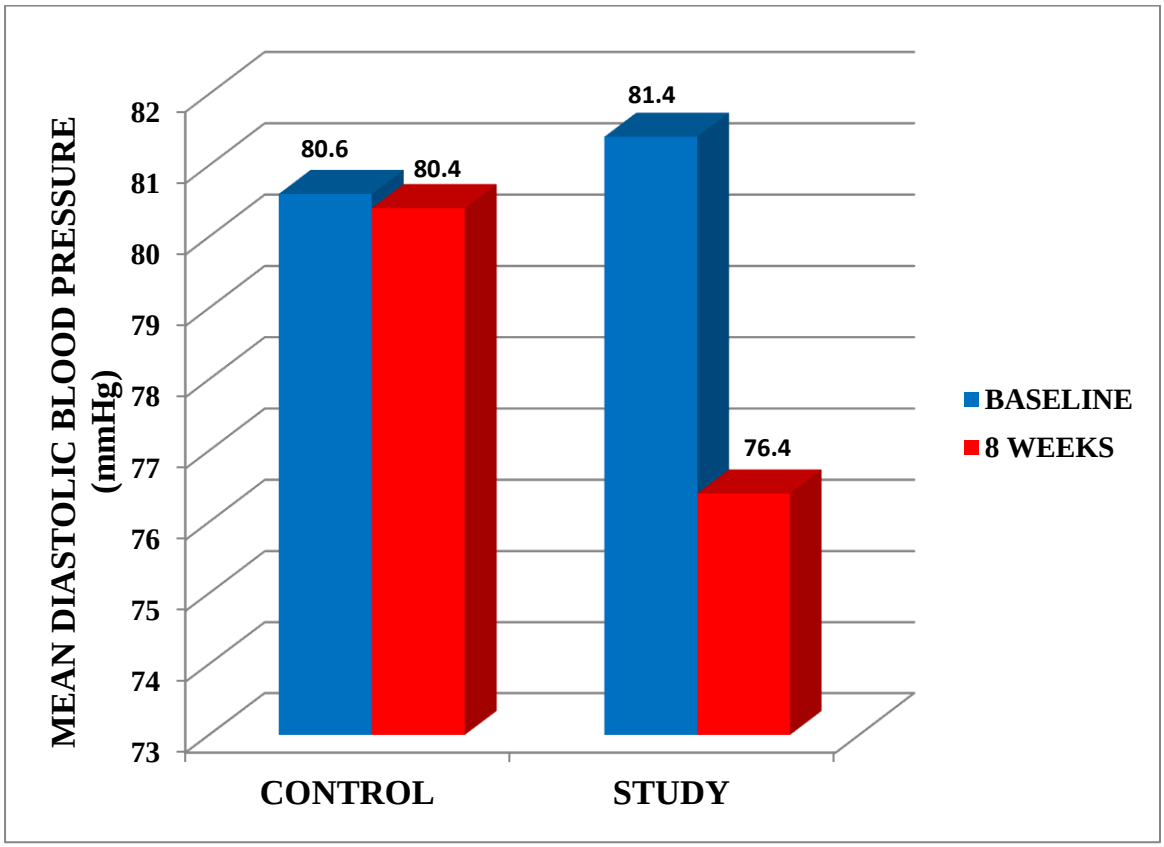


Fig 9 is a graphical representation of Table 9

TABLE 10: OTHER BIOCHEMICAL INVESTIGATIONS

PARAMETER	GROUP A (CONTROL)			GROUP B (STUDY)		
	Day 0 (MEAN)	AT THE END OF 8WEEKS (MEAN)	P VALUE	DAY 0 (MEAN)	AT THE END OF 8 WEEKS (MEAN)	P VALUE
SGOT	24.87	25	0.894	25.1	24.9	0.86
SGPT	27.57	27.86	0.814	27.1	27.4	0.96
BILIRUBIN	0.86	0.85	0.946	0.86	0.83	0.54
SERUM UREA	28.36	29	0.967	26.93	27.13	0.87
SERUM CREATININE	0.893	0.884	0.938	0.91	0.87	0.69

Table 10- shows the biochemical investigations on Day 0 and at the end of 8 weeks in control and study groups. The differences in lab parameters were not statistically significant in both the groups.

TABLE 11: ADVERSE EVENT PROFILE

ADVERSE EVENT	CONTROL GROUP	STUDY GROUP
NAUSEA	3	2
VOMITING	1	0
ABDOMINAL PAIN	1	0
HYPOGLYCEMIA	3	2
METALLIC TASTE	2	0
DIZZINESS	0	1

FIGURE 11: ADVERSE EVENT PROFILE

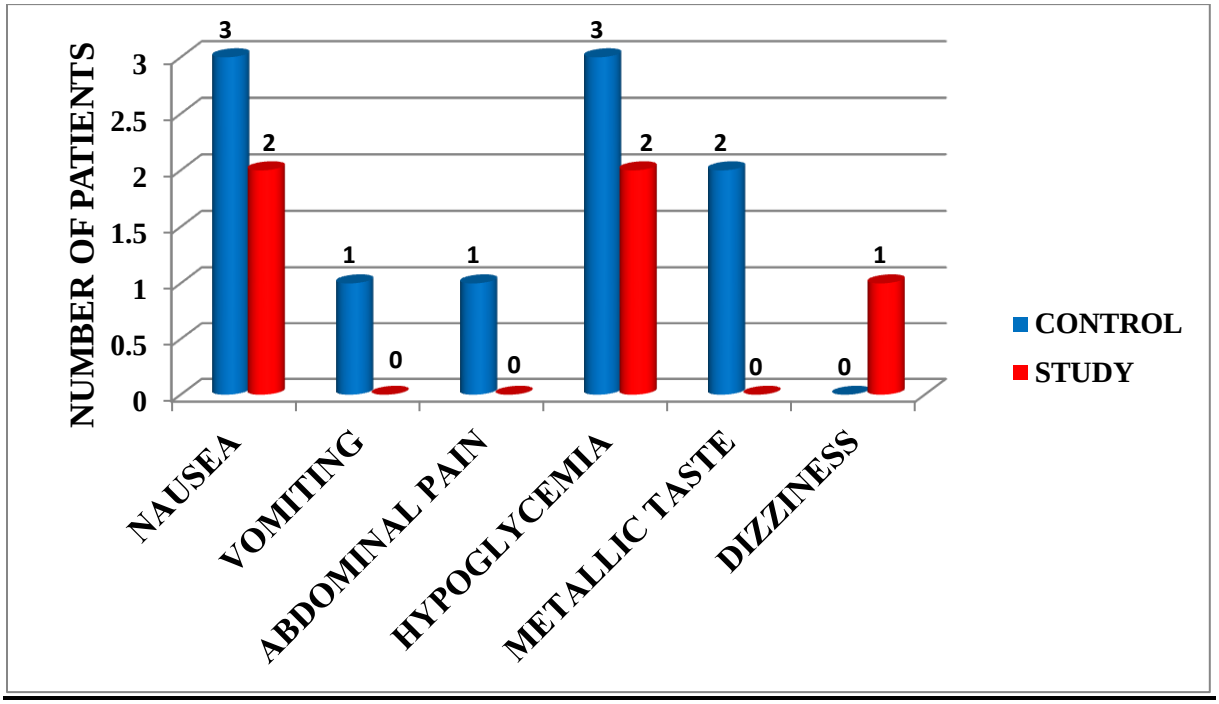


Fig 11 is a graphical representation of Table 11.

TABLE 12: INCIDENCE OF ADR'S

	CONTROL GROUP (n=30)	STUDY GROUP (n=30)
NO. OF ADR's	10 (33%)	5 (17%)

Table 13 shows the incidence of ADR's among patients in control and study groups which is 10 (33%) and 5 (17%) in the control and study groups respectively.

FIGURE 12: INCIDENCE OF ADR's

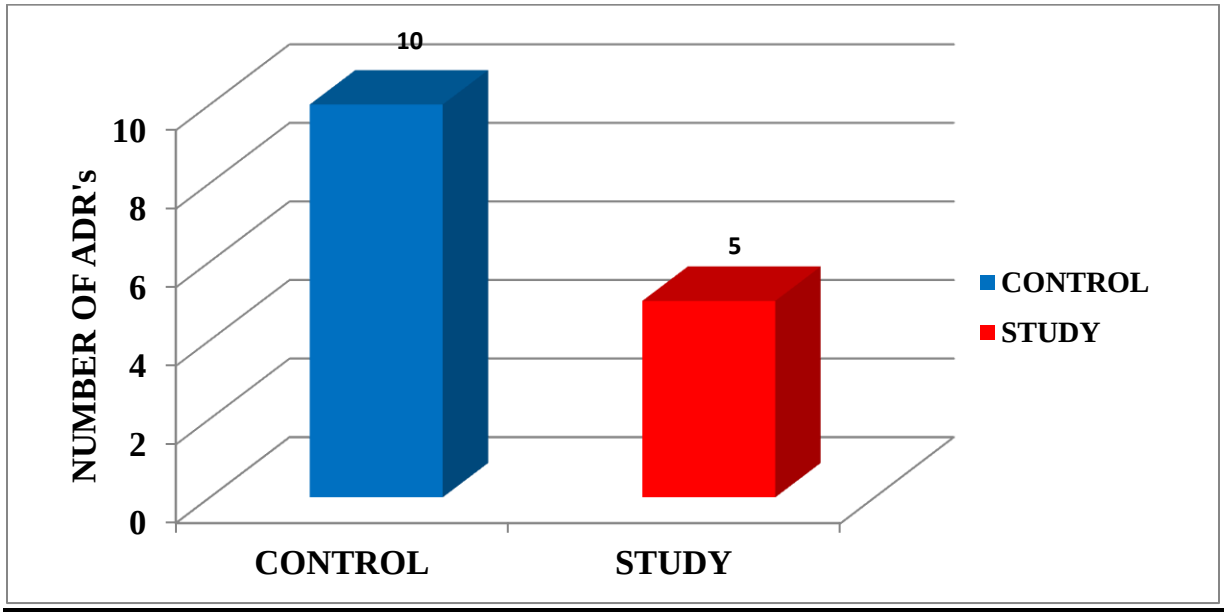


Fig 12 is a graphical representation of Table 12.

FIGURE 13: MEAN FASTING BLOOD GLUCOSE DURING STUDY AND FOLLOW UP PERIOD

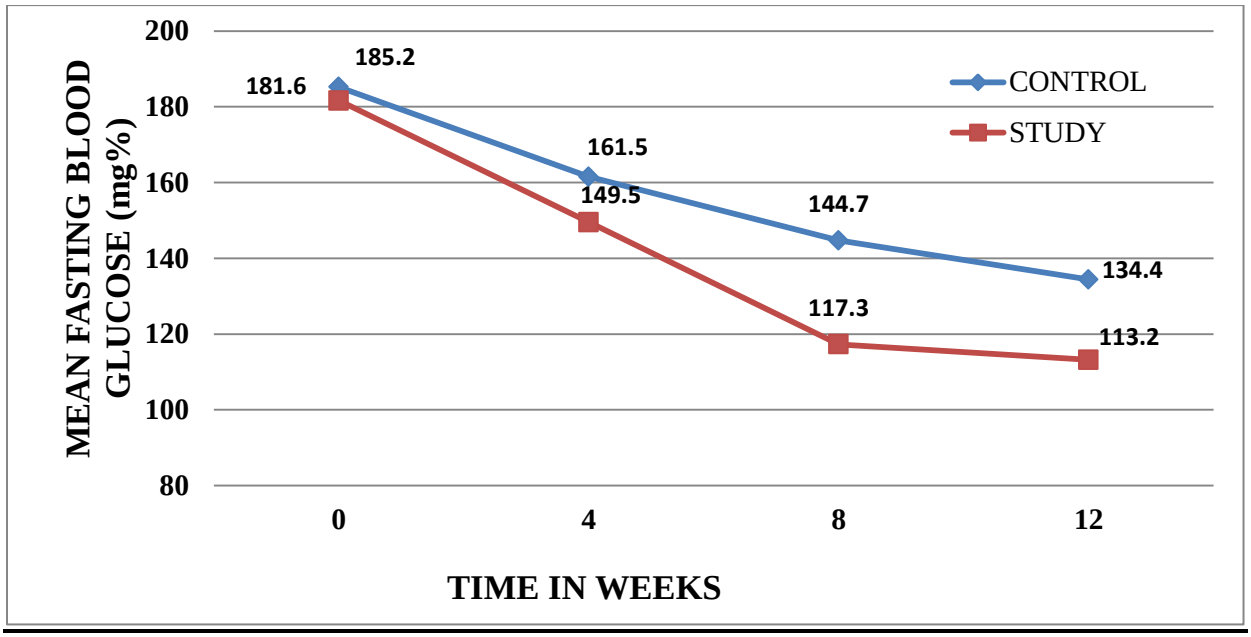


FIGURE 14- MEAN PERCENTAGE OF CRENATED RBCs WITH HEINZ BODIES DURING STUDY AND FOLLOW UP PERIOD

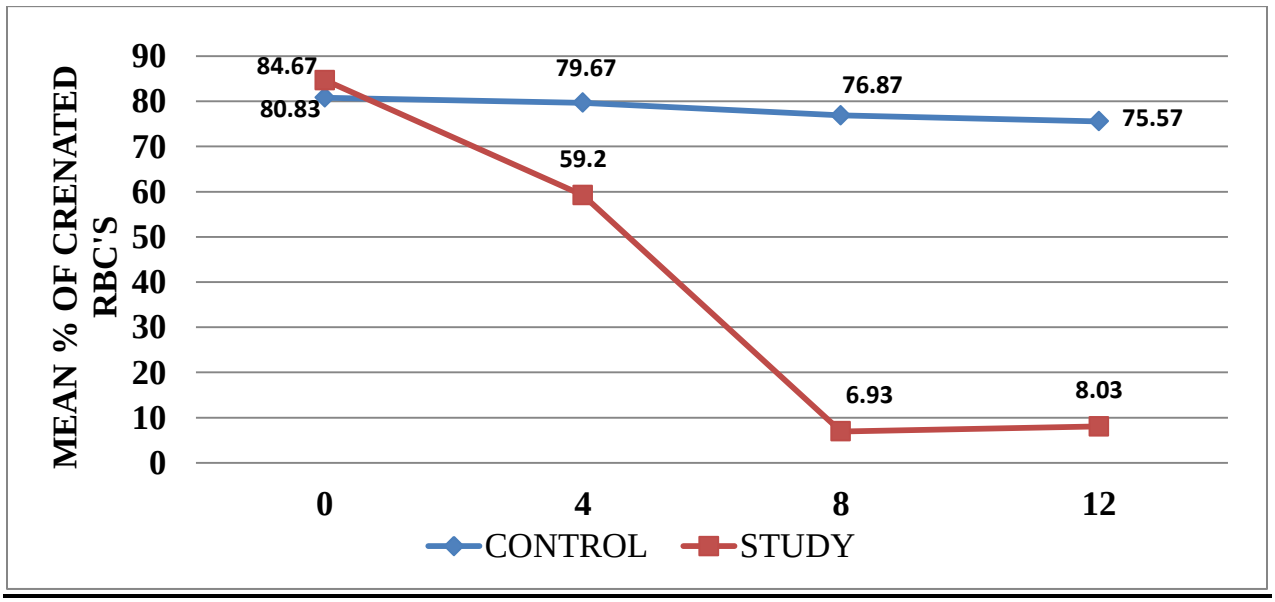


FIGURE 15: MEAN HAEMOGLOBIN DURING STUDY AND FOLLOW UP PERIOD

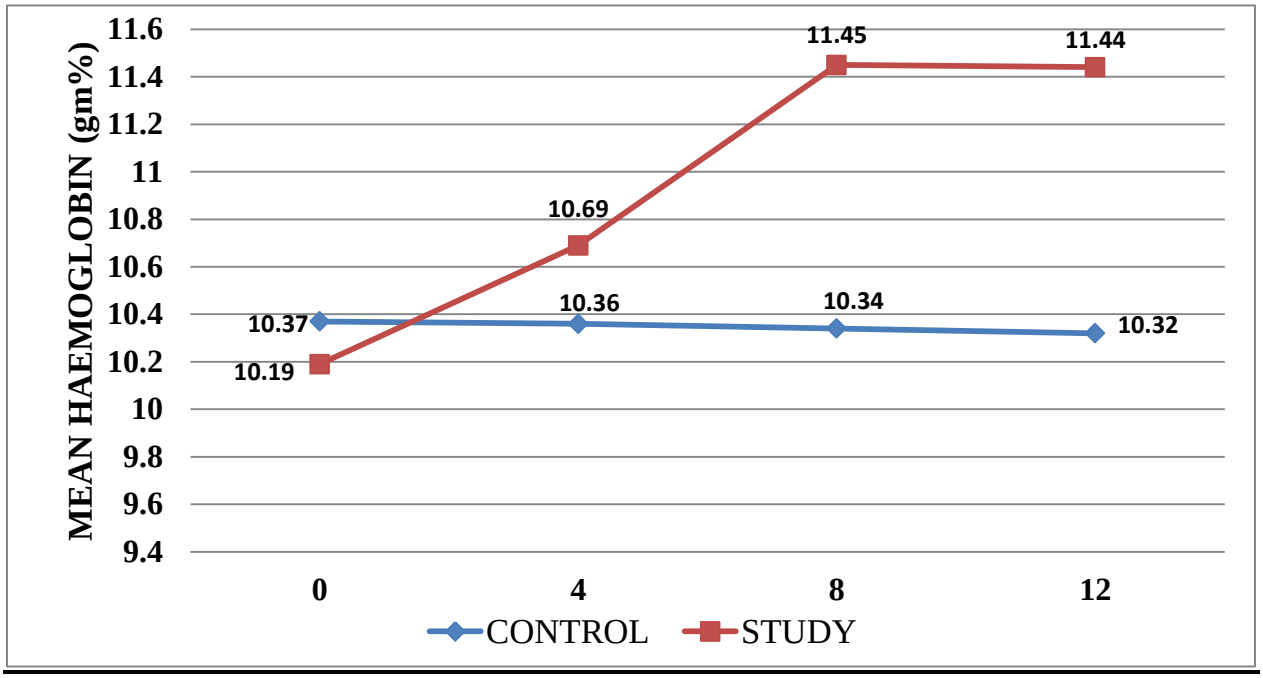
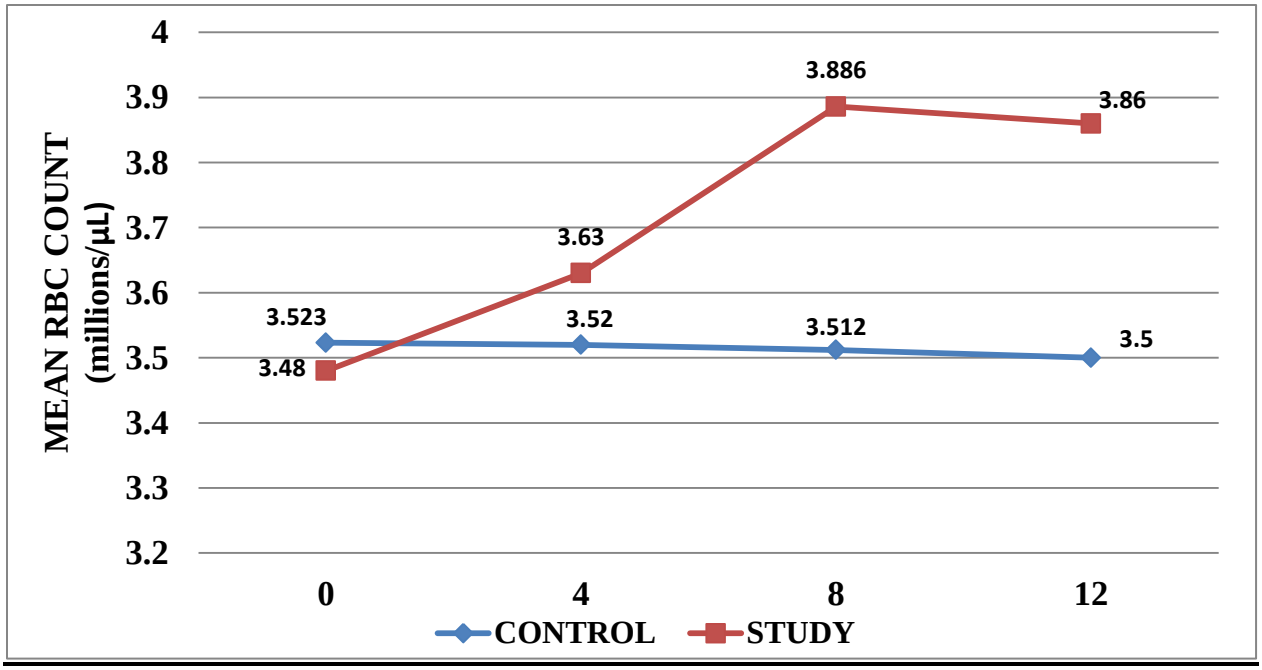


FIGURE 16: MEAN RED BLOOD CELL COUNT DURING STUDY AND FOLLOW UP PERIOD



DISCUSSION

DISCUSSION

Anemia is very common in many chronic inflammatory diseases. The prevalence of anemia in Type 2 diabetes is around 25% according to WHO guidelines.[85] Anemia is an important risk factor in diabetes causing micro- and macrovascular complications of diabetes mellitus like retinopathy, nephropathy, neuropathy and delayed wound healing. The cause of anemia in not recognized and patients are not treated in Type 2 diabetes. Early treatment of anemia is necessary so that the complications of diabetes and insulin resistance can be prevented.

Measuring hemoglobin% alone is not sufficient because the type of anemia cannot be assessed. The treatment of anemia varies with the nature of anemia. Therefore, a peripheral smear is necessary to find out the exact type of anemia.

In our study, we used cover slip technique to study the morphology of RBCs under high power microscope to find out the exact cause of anemia.

Oxidative stress plays a major role in the pathogenesis of Type 2 diabetes. Excess production of oxidants cause insulin resistance, RBC damage, tissue damage and various diabetic complications. [43]

RBCs are the first cells to be exposed to free radicals and therefore RBCs are susceptible to oxidative damage leading to cell membrane defects (crenated edges) and oxidation of hemoglobin forming Heinz bodies. [12]

The lifespan of RBCs is reduced leading to hemolytic anemia. The oxygen carrying capacity of hemoglobin is also reduced. The RBCs lose their shape and flexibility, reducing the microvascular circulation and causing various complications like retinopathy, nephropathy, neuropathy and non healing of wounds.

Antioxidants like α-tocopherol (Vitamin E) and ascorbic acid (Vitamin C) scavenge the free radicals, protect the cell membrane from oxidative damage and prevent peroxidation of lipids. They also reduce insulin resistance by activating tyrosine phosphorylation.[69] Therefore, treatment of anemia with antioxidants like Vitamin E and C in Type 2 diabetes protects the RBCs from oxidative damage, decrease insulin resistance and improves glycemic control.

In this study, 102 patients with early Type 2 diabetes (less than 2 years duration) were screened, 42 were excluded for various reasons and 60 patients were randomized by simple randomization into control and study groups of 30 patients each. The patients in the control group were on metformin alone while those in the study group received 400IU of Vitamin E and 500mg of Vitamin C once daily along with metformin for 8 weeks duration. Both the groups were followed up for 4 weeks post treatment.

The level of oxidative stress was assessed on the basis of the percentage of crenated RBCs with Heinz bodies along with the hemoglobin level and total RBC count.

Other parameters assessed were fasting blood glucose, systolic and diastolic blood pressure.

Among the 60 patients who completed the study, the mean age was 48 years and 47 years in the control and study groups respectively. This showed that most of the patients were middle aged.

There were a higher proportion of female subjects in both the control (67%) and study (63%) groups.

The mean duration of diabetes was 11 months in control and 9 months in the study group.

There was no significant difference in any of the parameters assessed at baseline between the control and study groups.

At the end of the 8 week study period, the mean fasting blood glucose was lower in the study group ($p=0.001$) compared to the control group ($p=0.030$). This showed that adding antioxidants to the standard regimen resulted in decrease in insulin resistance and a better control of blood glucose levels.

Nearly 80% of the RBCs in both the groups showed crenated edges with Heinz bodies at the beginning of the study. This indicates the degree of ongoing hemolysis in patients with Type 2 diabetes. At the end of 8 weeks, the percentage (%) of crenated RBCs with

Heinz bodies was reduced significantly to 6.9% in the study group (p=0.001) and to only 76% in control group (p=0.051). This clearly demonstrates that treatment with antioxidants improves membrane integrity and reduces RBC fragility and hemolysis by preventing free radical induced oxidative damage.

The mean hemoglobin was below average in both the control (10.37gm/dl) and study (10.1gm/dl) groups. After 8weeks of treatment, the study group showed a significant increase in hemoglobin (11.45 gm/dl) compared to the control group (10.34gm/dl).

This proves that anemia in Type 2 diabetes is due to hemolysis induced by oxidative damage to RBCs.

The mean total RBC count was also significantly increased in the study group compared to the control group (p=0.003).

The mean blood pressure was slightly above average in both the groups at baseline. At the end of 8 weeks there was a significant reduction in systolic (p=0.042) and diastolic (p=0.035) blood pressure in study group compared to control group. This may be attributed to the improvement in microcirculation and reduction in total peripheral resistance.[74]

There was a reduced incidence of adverse effects like nausea, vomiting, abdominal pain and hypoglycemia in the study group.

All the above mentioned effects were sustained in the study group at the end of the 4 week follow up period indicating that the beneficial effects of antioxidants persist for sometime even after its withdrawal.

In this study, the improvement in RBC morphology correlated well with the improvements in all other parameters like hemoglobin, blood glucose and blood pressure due to antioxidant therapy (Vitamin E & C),thus confirming the role of free radicals in the pathogenesis of Type 2 diabetes, anemia and its complications.

CONCLUSION

CONCLUSION

The prevalence of anemia in diabetes was 25% according to WHO guidelines whereas in our study anemia was above 80%.

Treatment of anemia in Type 2 diabetes is not included in the standard regimen. The type of anemia also was not given importance.

In this study, it was proved that the anemia is hemolytic in nature caused by oxidative stress. Treatment with antioxidants like Vitamin E and C significantly improved the hemoglobin %, RBC morphology and glycemic control in Type 2 diabetes.

The overall sense of well being was observed in these patients along with significant reduction in blood pressure. This proves that stress plays a major role in the pathogenesis of Type 2 diabetes and hypertension and antioxidants regulate the sympathetic nervous system.

Treatment of Type 2 diabetes with Antioxidants like Vitamin E and C can arrest the disease process, improve glycemic control and prevent the complications of diabetes. Using RBC morphology as a marker for oxidative stress is a simple, cost effective and novel investigation.

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APPENDICES

RED BLOOD CELL MORPHOLOGY AS A MARKER OF OXIDATIVE STRESS IN EARLY TYPE 2 DIABETES PATIENTS AND EFFICACY OF ANTIOXIDANTS AS AN ADD ON THERAPY TO STANDARD TREATMENT - A RANDOMIZED, OPEN LABEL, COMPARATIVE PILOT STUDY

CASE REPORT FORM

NAME: AGE/SEX : PLACE:

OP No: DIAGNOSIS:

Inclusion criteria: **YES/NO**

- Age: 30-70 years
- Sex-both genders
- Patients diagnosed with Type 2 Diabetes Mellitus within 2 yrs
- Patients on Metformin monotherapy.
- Patients willing to give written informed consent

EXCLUSION CRITERIA:

- Patients with Type 1 Diabetes Mellitus
- Current or ex smokers

- Pregnant and lactating women
- Patients with co-existing liver disease, heart disease, renal disease or malignancy
- Patients on any lipid lowering drugs
- Patients with any diagnosed hemotological disorder
- Patient enrolled in any other study

Subject initials: Subject number:

Subject: Included/Excluded Reason if excluded:

Informed Consent Obtained: Yes/No

CONTROL/ STUDY :

Signature of principal investigator

Visit 1

- Randomization done
- Vital signs recorded
- RBC morphology studied
- Fasting blood sugar, haemoglobin and RBC count measured
- Study medications issued for 4 weeks
- Instructed to return empty strips during subsequent visit

- Asked to report any adverse event, if any occurs.

Visit 2 (end of 4 weeks)

- Vital signs recorded
- Asked for return of empty strips to assess compliance
- Adverse events recorded, if any
- RBC morphology studied
- Fasting blood sugar, haemoglobin and RBC count measured
- Study medications issued for another 4 weeks

Visit 3 (end of 8 weeks)

- Vital signs recorded.
- RBC morphology studied
- Fasting blood sugar, haemoglobin and RBC count measured
- Adverse events recorded, if any

Visit 4 (end of 12 weeks)

- Vital signs recorded
- Adverse events recorded, if any
- RBC morphology studied
- Fasting blood sugar, haemoglobin and RBC count measured

INFORMATION TO PARTICIPANTS

Investigator:

Name of Participant:

Title:- **RED BLOOD CELL MORPHOLOGY AS A MARKER OF OXIDATIVE STRESS IN EARLY TYPE 2 DIABETES PATIENTS AND EFFICACY OF ANTIOXIDANTS AS AN ADD ON THERAPY TO STANDARD TREATMENT – A RANDOMIZED, OPEN LABEL, COMPARATIVE PILOT STUDY**

This study is conducted at Rajiv Gandhi Govt. General Hospital, Chennai. You are invited to take part in this study. The information in this document is meant to help you decide whether or not to take part. Please feel free to ask if you have any queries or concerns.

Purpose of this study

Diabetes is a common metabolic disorder characterized by hyperglycemia due to inadequate insulin levels or insulin resistance. A growing body of evidence shows that oxidative stress plays a key role in the pathogenesis of vascular changes in Diabetes. In this study we want to evaluate the effects of oxidative stress on the morphology of red blood cells in Diabetes and the role of Antioxidants in reversing these effects and in clinical improvement.

We have obtained permission from the Institutional Ethics Committee.

Study details

All patients in the study will be divided into 2 groups- A & B. You will be assigned to either of the groups. One group will receive the standard treatment & the other group will receive standard treatment + Antioxidants

Study Procedures

During this study, blood will be collected from you twice, once at start of treatment and the other at the end of the 8 week treatment period. The total amount of blood collected from you will not be more than 2ml. These samples will be used to evaluate your red blood cell morphology. You will be asked to come for follow up once after 4 weeks of completion of study. During the course of the study if you notice any adverse events, you have to report it. You will be required to return unused study medicines when you report for your scheduled visits. This will enable correct assessment of the study results.

Possible benefits to you – Antioxidants with your standard medications will reduce the level of oxidative stress and your future risk of developing complications due to Diabetes.

Possible benefits to other people - The results of the research may provide benefits to the society in terms of advancement of medical knowledge and/or therapeutic benefit to future patients.

Confidentiality of the information obtained from you

You have the right to confidentiality regarding the privacy of your medical information (personal details, results of physical examinations, investigations, and your medical history). By signing this document, you will be allowing the research team investigators, other study personnel, sponsors, Institutional Ethics Committee and any person or agency required by law like the Drug Controller General of India to view your data, if required.

The information from this study, if published in scientific journals or presented at scientific meetings, will not reveal your identity.

Participation and Withdrawal from the study

Your decision not to participate in this research study will not affect your medical care or your relationship with the investigator or the institution. You will be taken care of and you will not lose any benefits to which you are entitled. The participation in this research is purely voluntary and you have the right to withdraw from this study at any time during the course of the study without giving any reasons. However, it is advisable that you talk to the research team prior to stopping the treatment/discontinuing of procedures etc.

The results of this study will be informed to you at the end of the study.

Signature of Investigator Signature of Participant

Date Date

ஆய்வு தகவல் தாள்

ஆய்வு தலைப்பு :

இரத்த சிவப்பணுவின் அமைப்பில் ஏற்படும் மாற்றத்தின் மூலம் புதிதாக கண்டுபிடிக்கப்பட்ட இரண்டாம் வகை நீரிழிவு நோயின் போது ஏற்படக்கூடிய ஆக்ஸிடேட்டிவ் ஸ்டிரஸ்ஸை (ஆக்ஸிஜனேற்ற அழுத்தம்) கண்டறிதல் மற்றும் ஆக்ஸிடேட்டிவ் ஸ்டிரஸ்ஸை குறைப்பத்தில் விட்டமின்-E மற்றும் விட்டமின்-C யின் பங்கு வழக்கமான சிகிச்சை முறையுடன் ஓர் ஒப்பிடுதல் ஆய்வு.

ஆய்வாளர் :

பங்கேற்பாளர் :

இந்த ஆய்வு இராஜீவ் காந்தி அரசு பொது மருத்துவமனையில் நடைபெற உள்ளது. நீங்களும் இந்த ஆய்வில் பங்கேற்க நாங்கள் விரும்புகிறோம். இதிலுள்ள தகவலின் அடிப்படையில் இந்த ஆய்வில் பங்கேற்பதா அல்லது வேண்டாமா என்று நீங்கள் முடிவு செய்து கொள்ளலாம். உங்களது சந்தேகங்களை எங்களிடம் கேட்டு நிவர்த்தி செய்து கொள்ளலாம்.

இந்த ஆய்வில் நோக்கம்:

உடலின் இன்சுலின் ஹார்மோன் குறைவதால் அல்லது இன்சுலின் ஹார்மோன் செயலிழப்பினால் இரத்தத்தில் சர்க்கரையின் அளவு அதிகரித்து நீரிழிவு நோய் ஏற்படுகிறது. நிறைய ஆய்வுகளின்படி நீரிழிவினால் ஏற்படக்கூடிய பின்விளைவுகளுக்கு ஆக்ஸிடேட்டிவ் ஸ்டிரஸ் (ஆக்ஸிஜனேற்ற அழுத்தம்) முக்கியபங்கு வகிக்கிறது. இந்த ஆய்வில் ஆக்சிடேட்டிவ் ஸ்டிரஸ்ஸை இரத்த சிவப்பணுவின் அமைப்பில் ஏற்படும் மாற்றத்தின் மூலம் கண்டறிதல், மேலும் விட்டமின்கள்-E மற்றும்-C எவ்வாறு இதனை சரிசெய்ய பயன்படுகின்றன என்பதை கண்டறிதல்.

இந்த ஆய்விற்கு இன்ஸ்டிட்யூசனல் எத்திக்கல் கமிட்டி சம்மதம் பெற்றிருக்கிறோம்.

ஆய்வின் செயல்முறை:

இந்த ஆய்வில் கலந்துகொள்பவர்கள் A மற்றும் B என்று இரு குழுக்களாக பிரிக்கப்படுவீர். A குழுவில் இருப்பவர்கள் வழக்கமான சிகிச்சையும் B குழுவில் இருப்பவர்கள் வழக்கமான சிகிச்சையுடன் விட்டமின்-C மற்றும் விட்டமின்-E மருந்தும் பெறுவர்.

இந்த ஆய்வில் முதல் மற்றும் 8 வார முடிவில் இரத்த பரிசோதனை செய்யப்படும். அதற்காக எடுக்கப்படும் இரத்தத்தின் அளவு அதிகபட்சம் 2மி.லி. இந்த ஆய்வின் போது ஏதேனும் பக்க விளைவுகள் ஏற்பட்டால் உடனடியாக எங்களிடம் தெரிவிக்க வேண்டும். மேலும் நீங்கள் உபயோகப்படுத்தாத மாத்திரைகளை எங்களிடம் திரும்ப தருமாறு கேட்டுக் கொள்கிறோம்.

ஆய்வினால் ஏற்படும் நன்மைகள்:

இந்த ஆய்வில் கலந்துக் கொள்வதன் மூலம் நீங்கள் நோயின் தன்மையில் முன்னேற்றம் பெறலாம். மேலும் வருங்காலத்தில் பிறநோயாளிகளும் பயன்பெற இந்த ஆய்வு உதவியாக அமையும்.

மருத்துவ சிகிச்சையின் தகவல்கள் குறித்த விவரங்கள்:

உங்கள் மருத்துவ சிகிச்சை குறித்த தகவல்கள் ரகசியமாக பாதுகாக்கப்படும் (பெயர், மருத்துவ பரிசோதனை முடிவு, மருத்துவ ஆய்வு முடிவு) இந்த தகவல் தாளில் கையெழுத்திடுவதின் மூலம் உங்களை பற்றிய குறிப்புகளோ, எடுத்துக்கொண்ட சிகிச்சை முறையை பற்றியோ ஆய்வாளரோ இன்ஸ்டிட்யூசன் எத்திக்கல் கமிட்டியை சார்ந்தவர்களோ தேவைப்பட்டால் அறிந்து கொள்ளலாம் என்று சம்மதிக்கிறீர்கள். முடிவுகளை அல்லது கருத்துக்களை வெளியிடும் போதோ அல்லது ஆய்வின் போதோ தங்களது பெயரையோ அல்லது அடையாளங்களையோ வெளியிடமாட்டோம் என்பதையும் தெரிவித்துக் கொள்கிறோம்.

இந்த ஆய்வில் பங்கேற்காவிட்டாலும் நீங்கள் வழக்கமான சிகிச்சையை தொடர்ந்து பெறலாம்.

இந்த ஆய்வில் பங்கேற்பது தங்களுடைய விருப்பத்தின்பேரில்தான் இருக்கிறது. மேலும் நீங்கள் எந்நேரமும் இந்த ஆய்விலிருந்து பின்வாங்கலாம் என்பதையும் தெரிவித்துக் கொள்கிறோம்.

இந்த சிறப்பு சிகிச்சையின் முடிவுகளை ஆய்வின் போதோ அல்லது ஆய்வின் முடிவின் போதோ தங்களுக்கு அறிவிப்போம் என்பதையும் தெரிவித்துக் கொள்கிறோம்.

ஆய்வாளர் கையொப்பம் பங்கேற்பாளர் கையொப்பம்

தேதி :

INFORMED CONSENT FORM

Red blood cell morphology as a marker of oxidative stress in early Type 2 diabetes patients and efficacy of antioxidants as an add on therapy to standard treatment – a Randomized, Open label, Comparative Pilot study

Name of the Participant:

I _____ have read the information in this form (or it has been read to me). I was free to ask any questions and they have been answered. I am over 18 years of age and, exercising my free power of choice, hereby give my consent to be included as a participant in this study.

1. I have read and understood this consent form and the information provided to me.

2. I have had the consent document explained to me.

3. I have been explained about the nature of the study.

4. I have been explained about my rights and responsibilities by the investigator.

5. I am aware of the fact that I can opt out of the study at any time without having to give any reason and this will not affect my future treatment in this hospital.

6. I hereby give permission to the investigators to release the information obtained from me as result of participation in this study to the sponsors, regulatory authorities, Govt. agencies, and IEC.I understand that they are publicly presented.

7. I have understand that my identity will be kept confidential if my data are publicly presented

8. I have had my questions answered to my satisfaction.

9. I have decided to be in the research study.

I am aware that if I have any question during this study, I should contact the investigator.

By signing this consent form I attest that the information given in this document has been clearly explained to me and understood by me, I will be given a copy of this consent document.

1. Name and signature / thumb impression of the participant (or legal representative if participant incompetent)

Name _____ Signature_____ Date_____

2. Name and Signature of impartial witness (required for illiterate patients):

Name _____ Signature_____ Date_____

Address and contact number of the impartial witness:

Name and Signature of the investigator or his representative obtaining consent:

Name _____ Signature_____ Date_____

சுய ஒப்புதல் படிவம்

ஆய்வு தலைப்பு :

இரத்த சிவப்பணுவின் அமைப்பில் ஏற்படும் மாற்றத்தின் மூலம் புதிதாக கண்டுபிடிக்கப்பட்ட இரண்டாம் வகை நீரிழிவு நோயின் போது ஏற்படக்கூடிய ஆக்ஸிடேட்டிவ் ஸ்டிரஸ்ஸை (ஆக்ஸிஜனேற்ற அழுத்தம்) கண்டறிதல் மற்றும் ஆக்ஸிடேட்டிவ் ஸ்டிரஸ்ஸை குறைப்பதில் வைட்டமின்-E மற்றும் வைட்டமின்-C யின் பங்கு வழக்கமான சிகிச்சை முறையுடன் ஓர் ஒப்பிடுதல் ஆய்வு.

பெயர் : வயது : தேதி : உள்நோயாளி எண் :

..... என்பவராகிய நான் இந்த ஆய்வின் விவரங்களும் அதன் நோக்கங்களும் முழுமையாக அறிந்து கொண்டேன். எனது சந்தேகங்கள் அனைத்திற்கும் தகுந்த விளக்கம் அளிக்கப்பட்டது. இந்த ஆய்வில் முழு சுதந்திரத்துடன் மற்றும் சுயநினைவுடன் பங்கு கொள்ள சம்மதிக்கிறேன்.

எனக்கு விளக்கப்பட்ட விஷயங்களை நான் புரிந்து கொண்டு நான் எனது சம்மதத்தைத் தெரிவிக்கிறேன். இச்சுய ஒப்புதல் படிவத்தை பற்றி எனக்கு விளக்கப்பட்டது.

இந்த ஆய்வினை பற்றிய அனைத்து தகவல்களும் எனக்கு தெரிவிக்கப்பட்டது. இந்த ஆய்வில் எனது உரிமை மற்றும் பங்கினை பற்றி அறிந்து கொண்டேன்.

இந்த ஆய்வில் பிறரின் நிர்பந்தமின்றி என் சொந்த விருப்பத்தின் பேரில்தான் பங்கு பெறுகிறேன் மற்றும் நான் இந்த ஆராய்ச்சியிலிருந்து எந்நேரமும் பின் வாங்கலாம் என்பதையும் அதனால் எந்த பாதிப்பும் ஏற்படாது என்பதையும் நான் புரிந்து கொண்டேன்.

இந்த ஆய்வில் கலந்து கொள்வதன் மூலம் என்னிடம் பெறப்படும் தகவலை ஆய்வாளர் இன்ஸ்டிட்யூசனல் எத்திக்ஸ் கமிட்டியினரிடமோ, அரசு நிறுவனத்திடமோ தேவைப்பட்டால் பகிர்ந்து கொள்ளலாம் என சம்மதிக்கிறேன்.

இந்த ஆய்வின் முடிவுகளை வெளியிடும்போது எனது பெயரோ, அடையாளமோ வெளியிடப்பட்டாது என அறிந்து கொண்டேன். இந்த ஆய்வின் விவரங்களைக் கொண்ட தகவல் தாளைப் பெற்று கொண்டேன். இந்த ஆய்விற்காக இரத்தப் பரிசோதனை செய்துக் கொள்ள சம்மதிக்கிறேன்.

இந்த ஆய்வில் பங்கேற்கும் பொழுது ஏதேனும் சந்தேகம் ஏற்பட்டால், உடனே ஆய்வாளரை தொடர்பு கொள்ள வேண்டும் என அறிந்து கொண்டேன்.

இச்சுய ஒப்புதல் படிவத்தில் கையெழுத்திடுவதன் மூலம் இதிலுள்ள அனைத்து விஷயங்களும் எனக்கு தெளிவாக விளக்கப்பட்டது என்றும் தெரிவிக்கிறேன் என்று புரிந்து கொண்டேன். இச்சுய ஒப்புதல் படிவத்தின் ஒரு நகல் எனக்கு கொடுக்கப்படும் என்றும் தெரிந்து கொண்டேன்.

பங்கேற்பாளர் / பாதுகாவலர் கையொப்பம் தேதி :

ஆய்வாளர் கையொப்பம் தேதி :

@EJL 1284.4
@EJL

INSTITUTIONAL ETHICS COMMITTEE
MADRAS MEDICAL COLLEGE, CHENNAI-3

EC Reg No.ECR/270/Inst./TN/2013
Telephone No. 044 25305301
Fax : 044 25363970

CERTIFICATE OF APPROVAL

To
Dr. Rohini Ann Mathew
Postgraduate M.D.(Pharmacology)
Madras Medical College
Chennai – 600 003.

Dear Dr.Rohini Ann Mathew,

The Institutional Ethics Committee has considered your request and approved your study titled **"Red Blood Cell morphology as a marker of oxidative stress in early Type 2 Diabetes patients and efficacy of antioxidants as an add on therapy to standard treatment – a Randomized, Open label, Comparative Pilot study" No. 55012015.**

The following members of Ethics Committee were present in the meeting held on 20.01.2015 conducted at Madras Medical College, Chennai-3.

1. Dr.C.Rajendran, M.D.,	: Chairperson
2. Dr.R.Vimala, M.D., Dean, MMC, Ch-3	: Deputy Chairperson
3. Prof.B.Kalaiselvi, M.D., Vice-Principal, MMC, Ch-3	: Member Secretary
4. Prof.R.Nandini, M.D., Inst.of Pharmacology, MMC	: Member
5. Prof.P.Ragumani, M.S., Professor, Inst.of Surgery, MMC	: Member
6. Prof.Md.Ali, M.D., D.M., Prof. & HOD of Medl.G.E., MMC	: Member
7. Prof.Uma Shanthi, Director i/c, Inst.of O&G, Chennai-3	: Member
8. Prof.K.Ramadevi, Director, Inst.of Biochemistry, MMC	: Member
9. Prof.Saraswathy, M.D., Director, Pathology, MMC, Ch-3	: Member
10. Prof.S.G.Sivachidambaram, M.D., Director i/c, Inst.of Internal Medicine, MMC	: Member
11. Thiru S.Rameshkumar, Administrative Officer	: Lay Person
12. Thiru S.Govindasamy, B.A., B.L.,	: Lawyer
13. Tmt.Arnold Saulina, M.A., MSW.,	: Social Scientist

We approve the proposal to be conducted in its presented form.

The Institutional Ethics Committee expects to be informed about the progress of the study and SAE occurring in the course of the study, any changes in the protocol and patients information/informed consent and asks to be provided a copy of the final report.

Member Secretary, Ethics Committee

MEMBER SECRETARY
INSTITUTIONAL ETHICS COMMITTEE
MADRAS MEDICAL COLLEGE
600 003