Indian Journal of Clinical Biochemistry, 2009 / 24 (4) 324-342

REVIEW ARTICLE

REACTIVE OXYGEN SPECIES, REACTIVE NITROGEN SPECIES AND ANTIOXIDANTS IN ETIOPATHOGENESIS OF DIABETES MELLITUS TYPE-2

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ABSTRACT

Diabetes mellitus type-2 (DMT-2) is a hyperglycemic syndrome with several characteristic features. It continues to rise unabatedly in all pockets of the world, parallels with affluence and can be controlled but not cured. It has a definite involvement of genetic component but environmental factors play overwhelmingly dominant role in etiopathogenesis. Insulin resistance (IR) and obesity are singular instigators of DMT-2. The various events cause critical defects in insulin signaling cascade followed by beta-cell dysfunction. Over a period of time, numerous other metabolic aberrations develop, resulting in diabetic complications which could be both vascular (cardiovascular complications, nephropathy, neuropathy, retinopathy and embryopathy) or a-vascular (cataract and glaucoma etc). It has been proposed that all these abnormal events are initiated or activated by a common mechanism of superoxide anion, which is accompanied with generation of a variety of reactive oxygen species (ROS), reactive nitrogen specie (RNS) and resultant heightened oxidative stress (OS). Provoked OS causes IR and altered gene expressions. Hyperglycemia induces OS through multiple routes : a)stimulated polyol pathway where in ≤ 30% glucose can be diverted to sorbitol and fructose, b)increased transcription of genes for proinflammatory cytokines and plasminogen activator inhibitor-1 (PAI-1) c) activation of protein kinase-C (PKC) leading to several molecular changes d)increased synthesis of Advanced Glycation End Products (AGEs) e)changes in a receptor far AGEs and f) autooxidation of glucose with formation of ketoimines and AGEs. All these processes are accompanied with alteration in redox status, ROS, RNS and OS which trigger DMT-2 and its complications. Initial hurriedly planned and executed experimental and clinical studies showed promising results of antioxidant therapies, but recent studies indicate that excess intake/ supplement may have adverse outcomes including increased mortality. It is advocated that antioxidants should be given only if preexisting deficiency is present. Selection of antioxidant is another important aspect. Lastly but most importantly the impact of OS is not obligatory but facultative. As such only those diabetic patients will be benefited by antioxidant therapies that have impelling punch of prooxidants.

KEY WORDS

Diabetes mellitus, Hyperglycemia, Free radicals, Antioxidants, Oxidative stress, Insulin.

INTRODUCTION

Diabetes mellitus with an alarmingly rising incidence (1) is a cluster of abnormal metabolic paradigm having a common feature of hyperglycemia (2-5) (Fig 1). Broadly it is classified into three categories: type-1 (≤ 10%), type-2 (80-90% DMT-2) and others (genetic defects of beta-cell, insulin processing and functioning, exocrine and endocrine defects, drug induced aberrations and gestational diabetes) which are < 10%. The prime concern in diabetes mellitus type-2 (DMT-2) is for several reasons, first, its incidence is relatively very high as compared to other types of diabetes, second, its incidence is exponentially rising and so far all measures have failed to curtail its rising incidence and deaths, third, increasing incidence of DMT-2 in children and adolescents, fourth, overwhelming participation

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of environmental factors which may act independently or in concert with genetic disposition, fifth, in due course of time a large number of DMT-2 patients develop multiple macro- or micro-vascular complications or both, sixth, the new molecular mechanisms involved in pathogenesis of the disease are now emerging, are more vigorously investigated and their interaction among themselves and their superimposition or intertwining with genetic factors are being critically evaluated, yet the etiopathogenesis of the disease is not fully understood (6-19). Obviously, clinicians have limited armamentarium for the treatment of the disease, and so far no drug is known to cure it permanently.

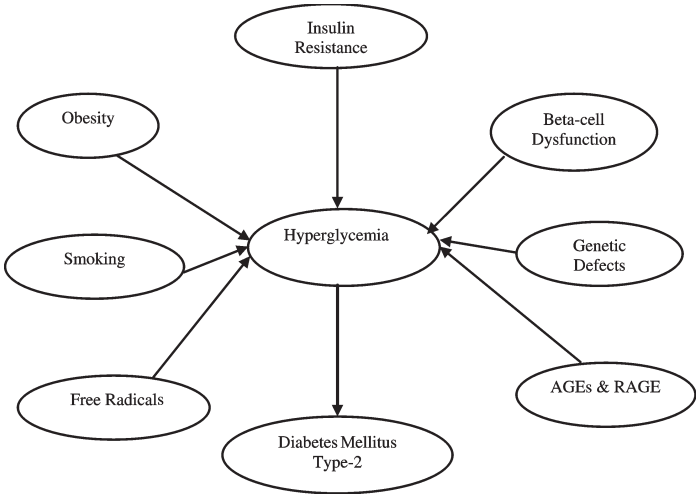

Fig 1 : Various defects leading to hyperglycemia and diabetes mellitus type-2

The atlas released by International Diabetes Federation in this month only (October, 2009) presents a disappointing global picture of the disease and still more disconcerting scenario for South-East Asia including India (1). Current estimates projected for India indicate that 7.1% adult population or 58.7 million people will have DMT-2 in 2010 as compared to 31.7 million in 2000 and this number will inflate by 12 million more patients in another 20 years. Adding insult to injury is the disquieting problem of the development of serious side complications over a period of time such as cardiovascular complications, retinopathy, nephropathy and embryopathy. Even the best control of the diabetes does not guarantee against the development of these complications and that these complications set faster in uncontrolled diabetes (2).

Induced hyperglycemia is an essential feature of the diabetes but is not the cause of the disease but consequence. Insulin secreted by beta-cells of Langerhans in pancreas, is the key hormone in the regulation of blood and tissue glucose homeostasis. Among the cluster of risk cascade in diabetes are obesity (20-22), insulin resistance (23-27), beta-cell dysfunction (28-29), formation of AGEs and RAGEs, auto-oxidation of glucose, activation of protein kinase C, polyol pathway, hexosamine pathway, numerous intracellular metabolic disturbances (30-32) smoking (33-37) and genetic disposition (2-12). Many of these abnormalities are intricately interconnected. What one has to clearly understand that the initial defect in DMT-2 does not lie in insulin secretion and function but else where in the remote areas of the body, especially peripheral tissue and adipose tissue who are main consumers of glucose. It is being argued that many of these defects either at site of origin or elsewhere are mediated by reactive oxygen species (ROS), reactive nitrogen species (RNs) from different sources (Fig 2). The proposed mechanism of involvement of oxidative stress (OS) in DMT-2 is depicted in Fig 3.

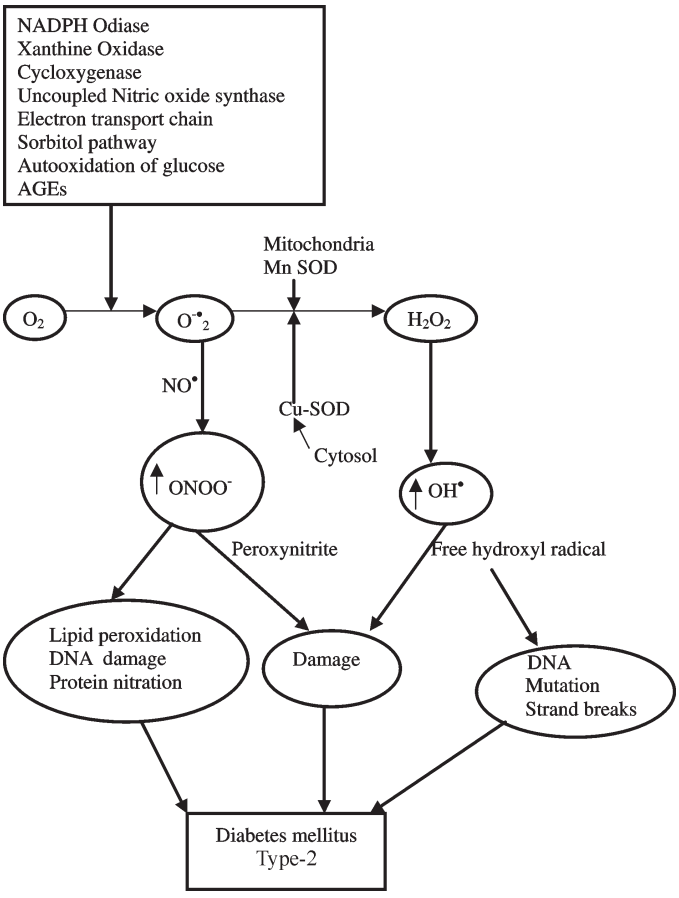

Fig 2 : Composite diagram showing different sources leading to enhanced generation of ROS in diabetes

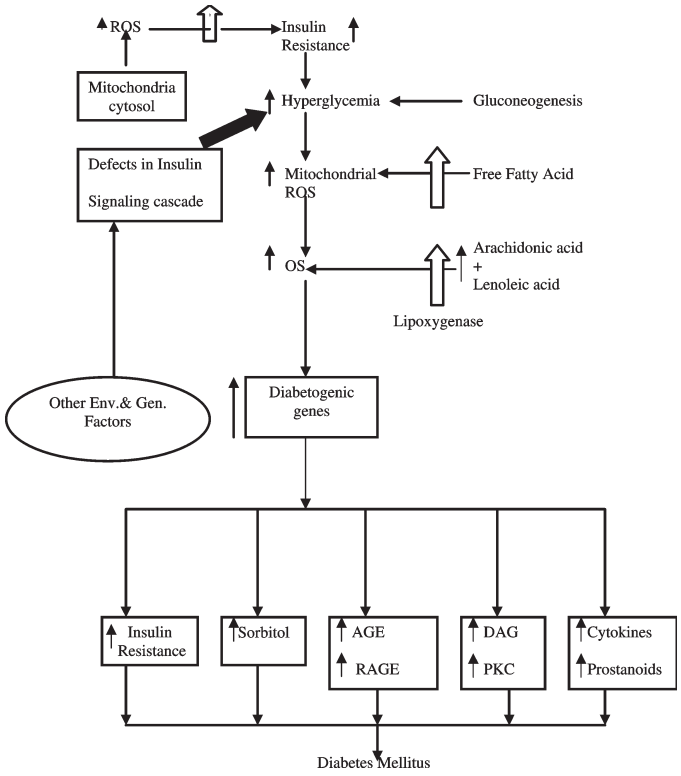

Fig 3 : Proposed mechanism for the involvement of oxidative stress in diabetes mellitus

The problem begins when influx of glucose in insulin dependent glucose utilizing cells and adipose tissue starts diminishing. For a considerable period of time, which may be as long as 10-20 years, beta-cells may compensate this defect by synthesizing more insulin and sending it in circulation to maintain normal level of glucose in blood and tissues, i.e. in the first instance hyperinsulinemia develops to compensate hyperglycemia. Progressively, beta-cells start getting tired and exhausted and level of insulin secretion starts declining with the result hypoinsulinemia develops along with overt DMT-2 (2, 9, 38). In the next phase, beta-cell dysfunction develops (17, 18, 38-40). Cells in various tissues develop metabolic stress of various types with varying magnitude which may culminate in diabetic complications (12, 16, 41-46). A diabetic person can develop one or more types of complications (2, 16). The risk factors can be divided into two groups, first, which initiate diabetic risk and second the intervening risk factors which join later and abet the progression of diabetes and diabetic complications. Thus DMT-2 is multifactorial, multimechanistic and multifaceted disease. In brief, several diverse mechanisms converge to cause DMT-2 and many of these and some of the supervening mechanisms ramify to cause complications (47). In the recent years an attractive hypothesis has developed, albeit not completely proven, that ROS and RNS could be a decisive risk force in the initiation of DMT-2 especially through insulin resistance (27) and obesity (21) and in development of diabetic complications due to chronic enraged OS (16). This chronic raised OS is reported to be due to several reasons such as increased formation of Advanced Glycation End Products (AGEs) and defect in a receptor for AGEs (RAGE), glucose autoxidation, increased diversion of glucose to sorbitol (polyol pathway) and hexosamine stimulated secretion of several proinflammatory cytokines, particularly Tumor Necrosis Factor Alpha (TNFα) and several other interleukins (Fig 3). The debate still continues on two points, first, whether OS can independently contribute to genesis of DMT-2 and second, is it hyperglycemia which raises OS that stimulates various pathways culminating in DMT-2.

ROS, RNS AND REDOX STATUS

Broadly ROS and RNS consist of free radicals and reactive species in these two groups and breakdown products of lipids proteins, nucleic acids and carbohydrates produced by them. Free radicals contain one or more unpaired electrons and could be positively or negatively charged or neutral in character. Superoxide anion ($O^{-\cdot}_2$), free hydroxyl radical ($\cdot OH$) and nitric oxide ($NO\cdot$) are important free radicals in human body and produce many other free radicals mainly from unsaturated fatty acids. They are unstable and aggressive molecules which have tendency to give its unpaired electron to other cellular molecules or snatch another electron from other molecules to attain stability. Physiologically they can be defined as overactive fragmented atoms or molecules which are capable of tormenting and fragmenting other molecules. Free hydroxyl is the most reactive neutral free radical with half life of about 10^{-9} second. It is capable of insulting fragmenting and mutating any cellular molecule with forceful intensity. Superoxide anion ($O^{-\cdot}_2$) in human body arises from metabolic reactions, irradiation and leakage from electron transport chain. Superoxide is often referred as primary ROS as most of other ROS and RNS arise from it and are therefore termed as secondary ROS and RNS. These free radicals are produced in cellular membrane mitochondria, nucleus, lysosomes, peroxisomes, endoplasmic reticulum and cytoplasm. The important non-radical species in human system are hydrogen peroxide, peroxynitrite, singlet oxygen peroxynitrous acid and peroxyl nitrite (15, 48-50). In low to moderate concentration they play important physiological role such as immunocompetance, apoptosis, vascular tone, hormonal regulation, signal transduction, transcription factors, defence genes and adaptive responses to enzymes (15, 50-53). However, in higher concentration they are harmful. Mounting

evidence suggests that they could be important players in the etiopathogenesis of age related diseases and other chronic inflammatory diseases (13, 15, 54, 55). Both ROS and RNS are reported to be involved in the etiopathogenesis of DMT-2.

The redox homeostasis is an essential phenomenon for normal health and survival of the cell and is defined as energy balance between prooxidizing and reducing environment for maintaining required concentration of electrons, known as redox state for proper functioning of the cell (15). The redox state may differ from cell to cell and tissue to tissue and is maintained within narrow limits. Many activities of the cell including several signaling systems are operated through changes in the redox state. Notably a 30 mV change due to changes in oxidant and reductant species in the cell may prove disastrous. FR help in maintaining this subtle redox balance. Increased FR activity increases prooxidizing environment which causes oxidation of biomolecules and decreased concentration of GSH. Altered redox status is reported to play a significant role in malfunctioning of signaling system and cell metabolism in many diseases including DMT-2. It is mainly attributed to aggravated production of $O^{-\cdot}_2$.

SOURCES FOR ENHANCED GENERATION OF $O^{-\cdot}_2$

A. Mitochondria : There are several sources for enhanced generation of $O^{-\cdot}_2$ in diabetes. The foremost source is electron transport chain. $O^{-\cdot}_2$ is produced at three points but under physiological conditions complex III is major site of $O^{-\cdot}_2$ generation (Fig 4). Long lived intermediates allow electrons with molecular oxygen to ultimately flow from water or $O^{-\cdot}_2$ but in DMT-2 the site shifts to complex-II with greater leakage of electron causing disturbed function of mitochondria. Infact many workers believe that mitochondrial dysfunction consequent to hyperglycemia is major factor in progression and development of DMT-2 and complications. Li et al (43) concluded from their studies that hyperglycemia induced mitochondrial ROS overgeneration was major molecular mechanism underlying the complications of the diabetes particularly diabetic nephropathy, which impairs endothelium dependent vasodilatation. Urocortin suppresses this ROS production. Interestingly experimental studies with Goto-Kakizaki rats have appeared both in favour and against this hypothesis. For example, Ferreria (56) demonstrated that rat liver mitochondria with adequate antioxidant activity decreased susceptibility to lipid peroxidation where as others (57) noted that different antioxidant approaches administered to rats had no effect in reversing the diabetic phenotype. In summary mitochondrial dysregulation is not prerequisite to initiation of DMT-2. This process sets in at some stage of disease process.

There are a number of other reports based on experimental studies implicating ROS as central mediators of DMT-2 progression and complications but not proven in humans. Further, raised OS is not an inevitable phenomenon in diabetes. More sophisticated experimental designs mimicking human situations should be designed. Further, to support the conclusions so obtained derived from them should be tested in clinical trials. Further, the problem does not end up here. We must have subtle tools and techniques to detect aberant mitochondria producing exacerbated quantity of ROS in diabetic patients because evidence available so far does not attest that raised OS is present in all cases. ROS are important players in this perturbation resulting in mitochondrial stress and development of diabetes and diabetic complications, if exacerbated.

Mootha et al (58) and Patti et al (59) have observed reduced expression of oxidative phosphorylation genes in DMT-2 with concomitant decreased expression of peroxisome coactivator I α (PUC-I α) in prediabetic and diabetic muscle. The exact mechanism linking exaggerated ROS production in mitochondria of diabetics is not fully understood but suggested hypothesis is that hyperglycemia increases electron transfer donors such as $FADH_2$ and NADH. Obviously it increases the electron flux on electron transport chain (ETC), thereby increasing ATP/ADP ratio and hyperpolarization of mitochondrial membrane potential. These process leads to high electrochemical potential difference, which partially inhibits ETC complex resulting in an accumulation of electrons to coenzymes Q causing enhanced concentration of reduced coenzyme Q. It is largely believed that this increased level of reduced coenzyme Q accompanied with accelerated production $O^{-\cdot}_2$ and subsequently several ROS are primarily responsible for mitochondrial dysfunction which play critical

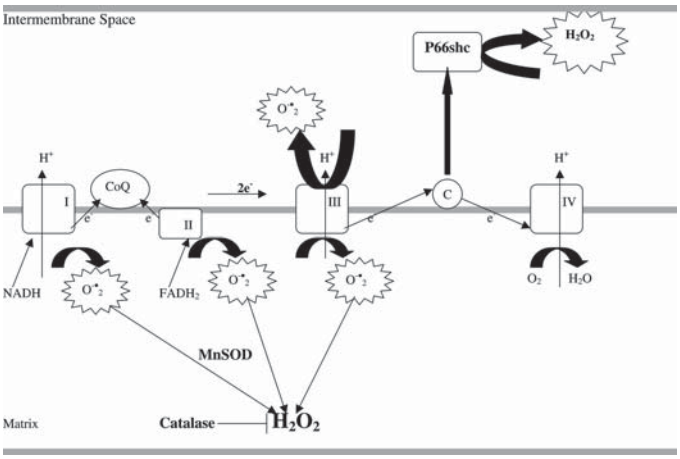

Fig 4 : Superoxide and hydrogen peroxide generation in mitochondria

role in progression of DMT-2 and if uncontrolled, initiate and accentuate diabetic complications (60-62).

B. NADPH Oxidases : NADPH oxidase is a multisubunit enzyme that catalyses $O^{-\cdot}_2$ formation by one electron reduction of O_2 using NADPH or NADH as electron donor requiring participation of Rac 2 or Racl and Rap-1A for activation (63-66)

$$2O_2 + NADPH\ (or\ NADH) \rightarrow 2\ O^{-\cdot}_2 + NADP\ (or\ NAD) + H^+$$

Initially NADPH oxidase was thought to be expressed only in phagocytic cells as a weapon generating ROS to confront and destroy invading bacteria. Now several forms of these enzymes have been discovered and expressed in many tissues and are designated as Knox family of NADPH oxidases. Enhanced generation of ROS due to increased expression of NADPH oxidases has been implicated in diabetes, atherosclerosis, hypertension, renal and neural diseases (15, 42, 67-69). Most importantly it has been implicated in development of various vascular complications in several disease conditions including DMT-2. The enzyme is responsive to several growth factors such as platelet-derived growth factor, epidermal growth factor, transforming growth factor beta, several cytokines (TNFα, interleukin I, platelet aggregation factor), G. Proteins-coupled receptor agonists (serotonin, thrombin, bradykinin, endothelin and angiotensin), free fatty acids, AGEs, RAGE, hyperglycemia and hyperinsulinemia. Angiotinsis II also influences enzyme activation through transcriptional regulation of oxidase subunits (68-72). NADPH oxidases are major source of glucose induced OS in kidney and arterial cells and can produce serious damage leading to diabetic vasculopathy and nephropathy (42, 67, 73). There is growing evidence that NADPH oxidases are significant contributor of diabetic hypertension and that these enzymes play a contributory role in the pathogenesis of inflammation, hypertrophy, endothelium dysfunction, apoptosis, migration and renal remodeling in hypertension, angiogenesis and DMT-2 (Fig 5) (42, 73). The effect of ROS on vascular cell growth, migration, proliferation and activation has been demonstrated by several workers (67,73). Notably, these diverse effects are mediated through redox sensitive regulation of multiple signaling molecules and second messengers including mitogen- activated protein kinases, protein tyrosine phosphatases, tyrosine kinases, proinflammatory genes, ionic calcium and ion channels.

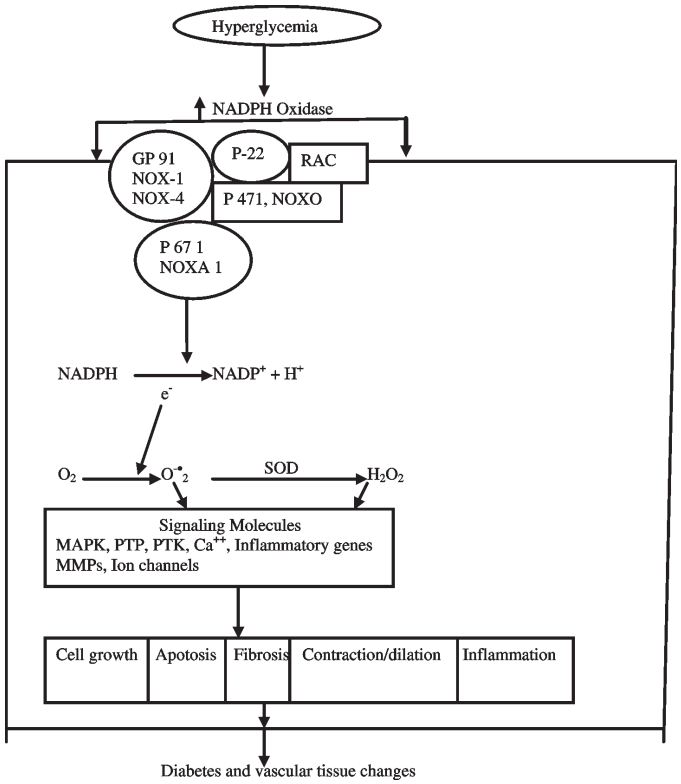

Fig 5 : Hyperglycemia induced NADPH oxidase activation in pathogenesis of diabetes and diabetic vasculopathy

C. Xanthine Oxidase : Xanthine oxidase catalyzes the conversion of hypoxanthine to xanthine and xanthine to uric acid and in both reactions $O^{-\cdot}_2$ is produced. The enzyme is derived from xanthine dehydrogenase. The over expression of xanthine oxidase results in raised OS. Allopurinol is an established drug to inhibit the activity of this enzyme and is commonly used to block the production of uric acid. The drug has been shown to reduce the OS in DMT-2 patients (15, 50, 73, 74). Experimental studies have also corroborated these findings. Some workers (74) have proposed that over expression of xanthine oxidase is a major contributor in DMT-2 but significant contribution of this enzyme in the raised OS in DMT-2 or diabetic complications is questionable.

D. Polyol Pathway : Conversion of glucose to sorbitol is known as polyol pathway. In the event of hyperglycemia intracellular glucose level rises. One of the options for the glucose is conversion to sorbitol by stimulation of enzyme aldose reductase and coenzyme NADPH. Depending upon the severity of hyperglycemia upto 30% of glucose can be diverted to polyol pathway. The excess diversion in this pathway has four disadvantages: a) excess production of sorbitol, b) deficiency or depletion of glutathione (GSH), c) excess production of ROS and d) excess production of AGEs. Intracellar accumulation of sorbitol is harmful. First, it causes cell damage, and second, it potently activates stress-sensitive signaling pathways including P3 MAPK and JNK. The

activation of this pathway has been shown to cause diabetic complications in transgenic mice with over expression of aldolase reductase and the inhibition of this enzyme retards or prevents development of neuropathy, nephropathy, retinopathy and cataract formation (42, 75-78). The excess of utilization of reduced NADP by aldose reductase may lead to deficiency or depletion of GSH which may have serious metabolic and physiologic consequences. However, some recent studies do not subscribe this view. Excess generation of ROS in this pathway has been detected but its quantitative contribution to ROS pool is not known. The most important aspect is excess formation of AGEs and RAGE which are undoubtedly participant in the diabetic complications (30, 79).

E. Hexosamine Pathway : Hyperglycemia stimulates hexosamine pathway through ROS in the endothelial cells abetting vascular complications in diabetes (15, 42, 80-82). Excessive glucose flux itself or by increasing free fatty acid level increase ROS which in turn stimulates amino-sugar formation from fructose in sorbitol pathway or mainly from the glucose itself (80,82). Activation of hexosamine pathway is also presumed to cause insulin resistance and promote late complications of the disease. The transgenic mice that over express glutamine: fructose-6-phosphate aminotransferase the rate limiting enzyme of this pathway, develops insulin resistance, hyperlipidemia and obesity. Hexosamine pathway also functions as a cellular sensor of energy availability and mediates the effect of glucose on the genes, inflammatory cytokines and plasminogen activator-I of several gene products. Obviously the over activation of hexosamine pathway in hyperglycemic condition promotes several complications.

F. Advanced Glycation End Products (AGEs) and Receptor for AGEs (RAGE) : AGEs constitute a multitude of non-enzymatically glycosylated proteins and lipids with altered biochemical and physiological properties. DMT-2 consequent to hyperglycemia leads to combination of protein and sugar unit in plasma and locally the site of vascular complications. The process is called protein glycation end products formation and products designated as Advanced Glycation End Products (AGEs) and are now establishedly recognized to exert several harmful effects (30,79). The process of protein glycation is initiated by a nucleophilic addition reaction between free amino group of protein and carbonyl group of a reducing sugar, especially glucose in human body. Initially Schiff's base in formed. The reaction is slow and takes several hours for completion and is reversible. As such reaction products upto this stage can be reversed to normal. However, so far no effects have been made to check the metabolic process at this point. Nor this task seems to be easy. In the second phase, as seen in DMT-2, the labile Schiff base reorganizes itself to nearly irreversible in a few days time. These are ketoamines and Amador products. This protein glycation depends on three conditions: a) degree and duration of hyperglycemia, b) permeability of glucose inside the cells and c) half life of protein i.e. proteins with greater longevity such as collagen are more prone to form AGEs. Strikingly the glycated proteins can further react with carbonyl intermediates such as deoxyglucosones to form the final products AGEs. More than a dozen AGEs have been detected in human tissues. They can induce serious molecular changes in other molecules. The raised levels of several AGEs like pentosidine, crossline and N- carboxymethyl lysine have been detected in DMT-2 and have closely been related to development and progression of diabetes and its complications: This route is not associated with ROS generation (Fig 6)

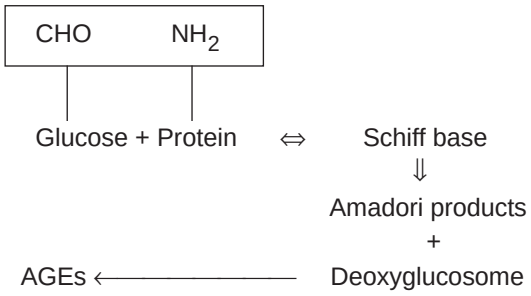

Fig 6 : Formation of AGEs without generation of ROS and RNS

The second pathway is known as glucose auto oxidation (83, 84) and generates ROS in the synthesis of AGEs (Fig 7). In vivo glucose is in equilibrium with its enediol form, though the latter is present only in small quantity. In the presence of transition metals, especially iron and copper, the enediols generate anion and themselves are oxidized to dicarbonyl ketoaldehydes which react with amino groups of proteins to from ketoimines. These ketoimines are more reactive than Amadori products, hence contribute greater share of AGEs than the first pathway. In the third pathway (Fig 8), Amadori products are formed which are finally converted to AGEs with protein enedios and protein dicarbonyl compounds. AGEs exert two harmful effects, first they themselves are toxic and second they exert toxic effects through ROS. Some of the toxic effects of AGEs are: a) ROS generation which can initiate lipid peroxidation, protein fragmentation and oxidation of nucleic acids resulting in alteration of the properties of these molecules. Further, their fragmented adducts may exert side toxic effects. b) they alter immunogenecity. Often antibodies are formed against AGEs in plasma and give birth to AGEs-Immune complex in DMT-2 patients and this promotes diabetes

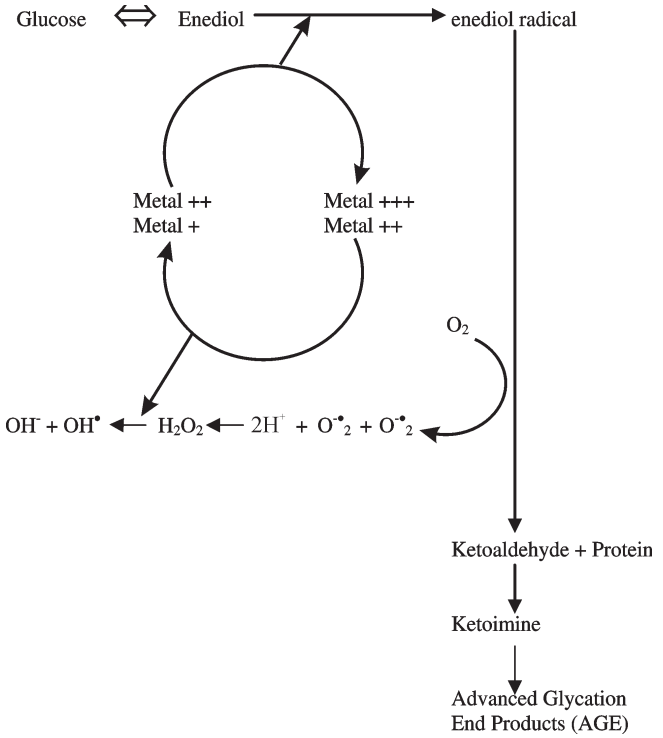

Fig 7 : Autooxidation of glucose resulting in oxidative stress through formation of superoxide anion ($O^{-\bullet}_2$) and free hydroxyl radical ($^{\bullet}OH$)

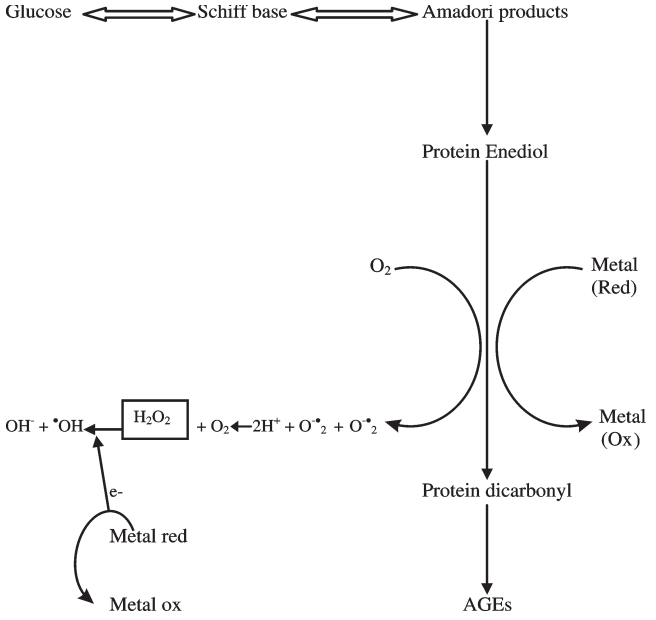

Fig 8 : Formation of AGEs, through protein carbonyl with generation of hydrogen peroxide and free radicals

and abets diabetic complications. c) they decrease ligand binding capacity d) modify half life of proteins e) adversely alter the activity of enzymes and f) nucleic acids and phospholipids become prone to glycation and may add new AGEs to its pre existing pool.

There are several types of cell receptors for AGEs (RAGEs) and one of them has specifically been related to pathogenesis of DMT-2. This RAGE is multiligand receptor and a member of super family of cell surface molecular receptors. It is present on macrophages, smooth muscle cells, endothelial cells and many other cells. This receptor interacts with several types of AGEs with particular affinity for AGE-N-Carboxymethyl lysine. The AGEs and proinflammatory cytokines stimulate RAGE. Their interaction is believed to initiate and aggravate diabetic complications. In addition they increase ROS generation in macrophages thereby causing heightened OS which in turn leads numerous changes which are particularly held responsible for chronic inflammatory conditions and other problems in DMT-2 (Fig 9) (30, 85-88).

In one of the excellent animal study Yan et al (89) demonstrated that infusion of AGEs was associated with heightened OS and activation of NF-kB and haem-oxygenase. The proposed mechanism of their involvement is that OS generated by AGE-RAGE interaction activates NF-kB which in turn modulates gene transcription for endthelin-1, thrombomodulin, extended generation of pro-inflammatory cytokines, enhanced expression of adhesion molecules, vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1. Finally they also increase vascular permeability. All these

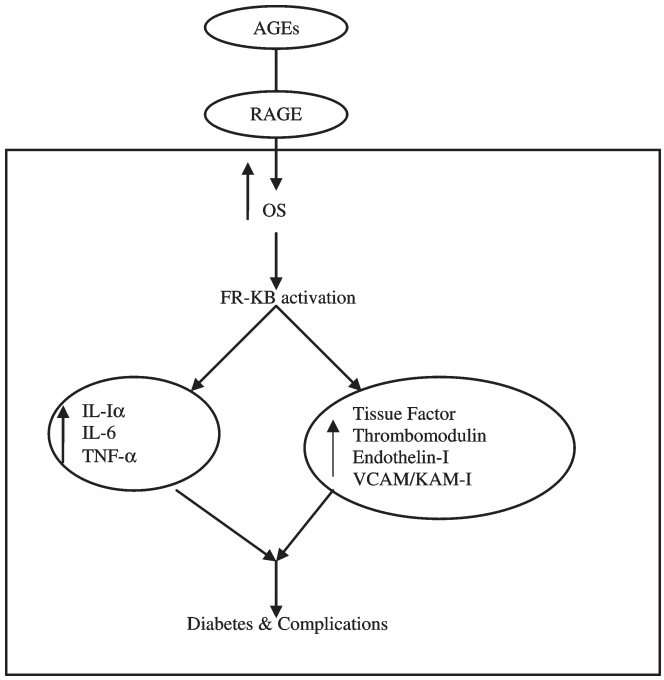

Fig 9 : AGE-RAGE induced DMT-2 and its complication through heightened OS

factors in tandem result in part or full blown development and progression of macro and micro vascular changes. Thus AGEs and RAGE are incriminated as significant risk factor in etiology of DMT-2 related atherosclerosis, myocardial infarction, stroke, neuropathy, embryopathy, nephropathy, retinopathy, cataract formation, delayed wound healing and many other metabolic and pathological changes. Further, experimental and cell culture studies have supported these claims. However, augmented OS is not an obligatory phenomenon. We have carried studies on diabetic patients in India and Nepal and in none of cohorts we have observed heightened OS as an inevitable phenomenon in all patients (12-14). As such, individual patient has to be checked for these changes, if a therapy has to be instituted for this purpose, because in the recent years therapeutics have been advocated as AGEs have emerged key substances in diabetic vascular remolding mediating impaired extracellular modification such as extracellular flexibility and increased matrix area by cross linking of matrix proteins through entrapping several molecules specially oxidized LDL, interaction and reducing the activity of matrix metalloproteins which may be involved in matrix degradation, cell migration, proliferation and survival. Brownlee has lucidly shown the aberrant molecular pathways (Fig 10).

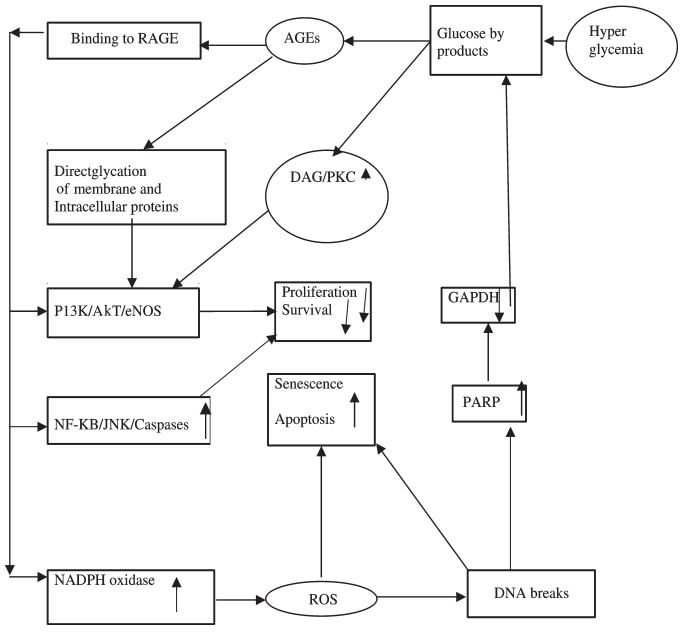

Fig 10 : Hyperglycemia induced pathways

Nitric Oxide

Nitric oxide is a free radical widely produced in body in macrophages, endothelial cells, neutrophils, hepatocytes, neuron and many other cells (15, 90-92) and can behave both as oxidant and antioxidant (91). It's important functions are relaxation of smooth muscles (vasodilatation), neurotransmitter, inhibition of adhesion, activation and aggregation of platelets, cytoprotection, signal transduction and antioxidant. However, when produced in excess, it causes toxicity promoting diabetic complications, atherosclerosis, neurotoxicity, increased adhesion, activation and aggregation of platelets and formation of another free radical nitrogen dioxide. For its versatile activity, it was named as the "Molecule of the Year" in 1992 by a science magazine (93) and fetched Nobel Prize to **Ferial Mural, Robert Furchgott and Lois Ignarrow** for their work on this molecule.

Nitric oxide can accept or donate electron depending upon physiological environment. It forms both nitric dioxide and peroxynitrite which are strong oxidizing agents. All of them have been associated in pathogenesis of diabetes. Nitric oxide is synthesized from arginine by three types of nitric oxide synthase: neuronal nitric oxide synthase (n-NOS), inducible nitric oxide synthase (inos) and endothelial nitric oxide synthase (eNOS). P 13 K signaling through Akt is a forceful prosurvival and proangiogenic mechanism. The signal stimulates phosphorylation and activation of e NOS, thereby generating nitric oxide for physiological activity. In diabetic patients e NOS becomes dysfunctional in blood vessel wall. This is due to decreased Akt activity, decreased phosphorylation and inactivation of eNOS via DAG/PKC pathway and oxidation of cofactor tertrahydrobiopterin. The process is referred as "e NOS uncoupling". This uncoupled e-NOS starts producing $O^{-\cdot}_2$ instead of nitric oxide, resulting in heightened OS and reduced nitric oxide levels (94). Spinetti et al (73) have concluded from diabetic models that control of OS and increased production of nitric oxide have beneficial effects in DMT-2 but success of such studies has yet to be attempted and proven in humans.

Insulin Resistance

Insulin resistance (IR) is defined as diminished response to normal concentration of insulin (23-25). A complex group of proteins of insulin signaling cascade are adversely affected and the major effect is on glucose uptake, metabolism and storage in peripheral and adipose tissues (27). It is a cardinal feature in DMT-2, present in about 75-80% patients and almost a universal feature in obese diabetics. The sequence of events are given in Fig 11. It is also present in other settings such as metabolic syndrome, cancer, cardiovascular diseases, burn trauma, cachexia, starvation, pregnancy conditions, but why IR occurs in so many contexts is poorly understood except in case of DMT-2 and obesity (95-97). The expression of IR differs in different conditions. It is initiated and triggered by both

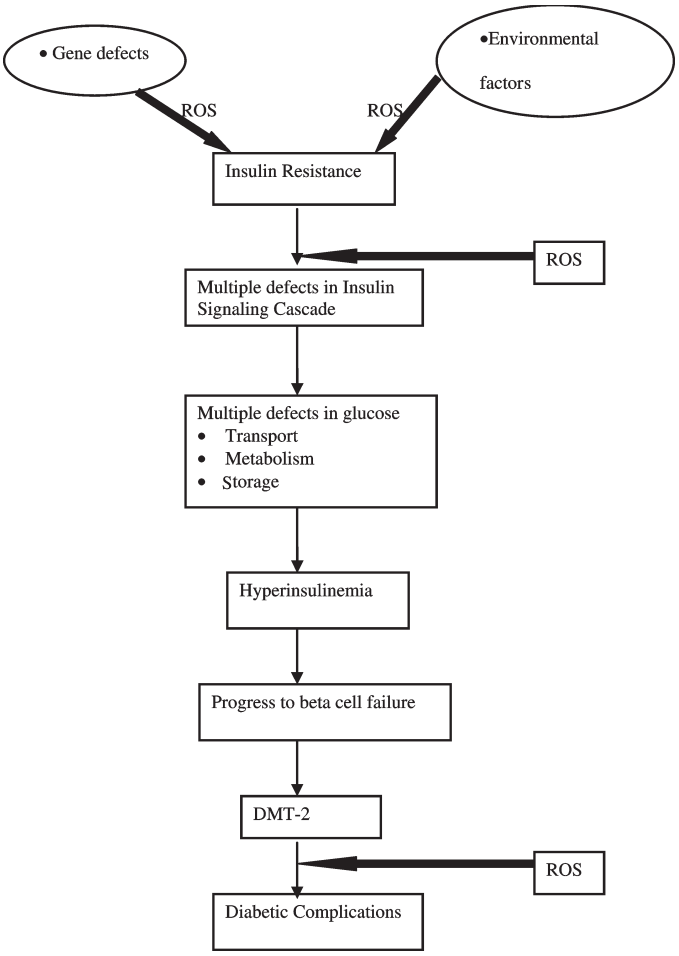

Fig 11 : ROS induced insulin resistance in causation of DMT-2

genetical and environmental factors where in the latter are overwhelmingly more important. Obesity is a central mediator of IR (97). The mechanistic defect in DMT-2 due to IR have received maximum attention where it primarily decreases the uptake of glucose in peripheral tissues.

The biochemical, physiological and pathological effects of insulin are now well established with two groups of scientists receiving Nobel Prizes for their contribution on insulin. However, it is agonizingly surprising that even today explicit molecular mechanisms of action of insulin and their control are still incompletely understood. The one action among numerous activities of insulin is the transport of glucose from extracellular fluid to inside peripheral muscle cells through GLUT-4 vesicles present inside these cells.

When insulin binds with its receptors on the surface of these cells, a series of reactions automatically proceed to activate signaling cascade leading to translocation, docking and fusion of GLUT-4 to cell surface. The GLUT-4 transporter has a gate, which then opens and glucose passes from outside to inside cell. When the requirement is over GLUT-4 detaches from the surface, floats inside the cell and gets encased again in a vesicle. IR interferes in this process. It induces two defects. First, the number of insulin receptors may decrease. This will result in decreased strength of insulin signaling message. Second, the GLUT-4 is unable to listen to the signal sent by insulin. Net result is decreased uptake of glucose by cell in peripheral tissues. To meet this exigency beta-cells of pancreas start sending more insulin. Obviously, hyperinsulinemia develops. The beta-cells have a limited capacity to adopt and over stretch to produce more insulin; beyond witch they not only fail to produce more insulin, but also lose their capacity do so thereby causing beta-cell dysfunction (15, 17, 18). IR may thus lead to hyperglycemia and overt diabetes in due course of time. What causes IR?

It has been argued that though there are several pathways which induce IR, the one significant source which causes IR is heightened OS. Many pathways operate through this source. However, it is still debatable whether OS is an obligatory cause of IR or not. Several workers believe that OS is consequence of hyperglycemia and this raised OS in turn may induce IR (18), but there could be other causes as well. For example about 25% of non-diabetic subjects may have IR in the range of that observed in DMT-2 but without raised OS. It deteriorates only when OS gets raised due to hyperglycemia. This raised activity of ROS could be pooled through several routes : a)elevated free fatty acids which encourage ROS production, b)uncoupling of electron transport chain, c) exaggerated production of ROS in mitochondrial dysfunction, d) potentiated glucose autoxidation, polyol and hexosamine pathways, e)stimulation of the synthesis of NADPH oxidase, xanthine oxidase and many other enzymes and f)enhanced production of non-enzymatic protein glycation, which may then as a secondary process exaggerate IR. Subsequently the IR so produced in tandem with other risk factors may be involved in the pathogenesis of DMT-2 on the contrary one of the most convincing experimental explanations about causal relationship of OS in IR has come from Houstis et al (27). They conducted cell culture studies and further supported them by animal experiments. In first instance they examined IR induced in 3T 3L-1 adipocytes by two treatments viz TNFα and dexamethasone. These two factors have been found to be associated with IR in several disease conditions but have quite different cellular response. TNFα is a cell surface receptor cytokine and exerts proinflammatory response. On the hand dexamethasone acts as nuclear hormone receptor with anti-inflammatory influence. However, both have common feature

to induce IR. Adipocytes developed IR within a few days of either of these treatments. To obtain optimal results of clinical and physiological relevance, the experimental protocol was designed to get: a) insulin dependent glucose uptake decrease by 50% which is frequently observed in DMT-2 and other conditions. b) insulin dependent uptake could be reversed by washing out either of the treatment and c) IR could be corrected by pioglitazone, a member of thiazolidinedione class of insulin sensitizing drugs. They analyzed results in two ways. The visual inspection distinctly showed ROS induced abnormality. These observations were further supported by gene set enrichment analysis. They observed that increased ROS levels precede the development of IR, that the level of protein carbonyl (marker of OS) increased 50% by TNFα or 110% by dexamethasone, and that when pioglitazone was coadministered with TNFα dexamethasone, the peroxidation processes were almost completely inhibited. They further demonstrated that n-acetylcysteine which stimulates synthesis of glutathione and tetrakis (4-benzoic acid) porphyrin which behaves as SOD and catalase exerted dose dependent decrease in IR. In summary all the observations taken collectivity indicated that suppression of ROS significantly reduced IR. Both TNFα and dexamethasone induced IR. This conclusion was further supported by experimental studies on oblob mouse. They proposed that ROS induced IR may be mediated through JNK which is known to be activated by OS. Both dexamethasone and TNFα raise JNK activity. The latter increasing mitochondrial ROS formation resulting in increased OS, which finally adversely affect insulin signaling cascade in IR. Since several pathways can lead to raised OS in diabetes, there is every likelihood IR could be net result of defects in several ROS producing routes. However, we are still not fully aware which ROS routes contributes to IR and what is magnitude of their contribution.

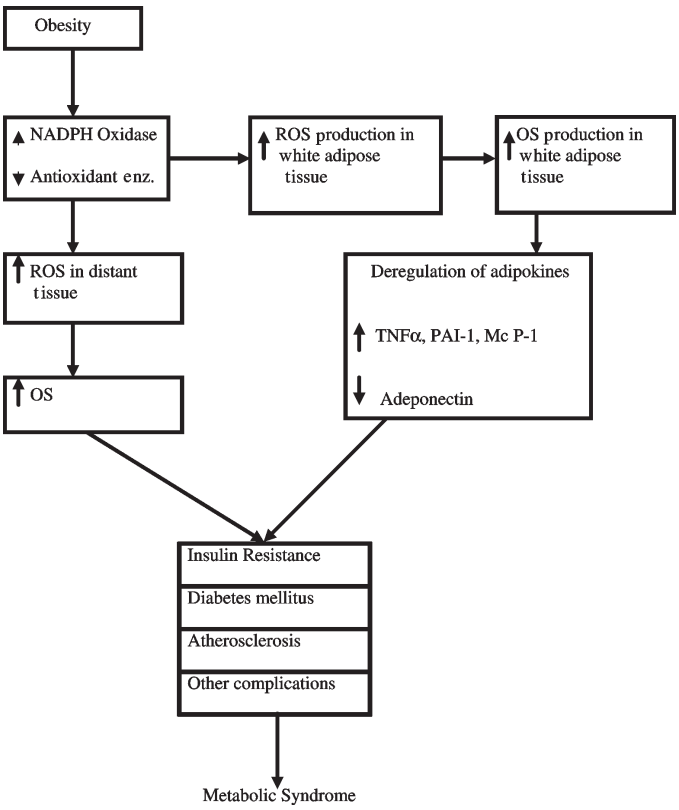

Fig 12 : Proposed mechanism for obesity induced ROS in the development of metabolic Syndrome

Obesity

Obesity is defined as 20% over the ideal weight. It is classified into two groups viz simple obesity and secondary obesity. Simple obesity is due to excess energy intake than expended. Secondary obesity is due to some associated pathology. Simple obesity is associated with atrocious outcomes and has almost espousal relationship with IR and DMT-2 (21, 22, 24). The surging wave of simple obesity is one of pressing health problems of our time as it leads to morbid and mortal conditions such as DMT-2, hypertension, cardiovascular diseases, neurological disorders, metabolic syndrome, cancer and many other complications including premature death (95-98). It can induce a cluster of risk conditions leading to as metabolic syndrome (Fig 12), which is threatening to be a global problem. The multihide of risk cascade is potentiated by the degree of obesity. Insulin plays a key role in energy homeostasis, lipogenesis and maintenance of redox status. It is also a central mediator of blood and tissue glucose homeostasis. Since it is the single and singularly potent hypoglycemic peptide hormone, human body has developed several switches and counter-switches to keeps its secretion and action under strict control. Simple obesity disturbs many of these switches through various pathways. Several of these pathways are reported to be mediated by raised OS which affects several signaling pathways and perturbs redox status towards more oxidizing environment. Putative evidence suggests that obesity is associated with chronic low grade inflammation and oxidative stress and is calamitous for health and scourge on mankind (99-100). The disproportionate rapid rise in prevalence of overweight and obesity in both developed and developing countries distinctly indicates that environmental changes are major determinants of this epidemic (21). Genetic disposition may act independently or in concert may abet the environmental factors (101). Many of these risk factors are reported to operate through ROS and RNS. Adverse effects of obesity have explicitly been linked with diabetes mellitus. Further there is gathering evidence that obesity also induces and/ or promotes a number of other diseases, and that it increases the chances of premature death (97,102). The

etiology of simple obesity is multifunctional and multimechanistic. The fundamental defect in this condition is lopsided energy management by body: more calories consumed than expended. Besides genetic inclination, the definite social, behavioral and environmental courses are increased consumptions of high energy foods particularly fast foods, increased frequency of food intake, indiscriminate selection of foods, especially refined foods, excess of trans fat and higher glycemic load carbohydrates. The social aspects influencing the caloric intake are overzealous advertising of sweetened beverages and foods. All these factors in concert tend to alter metabolism and appetite due to aforesaid reasons resulting in the tilted activities and excess energy intake, the body thus starts gaining weight. This gain is practically confined to accumulation of fat in adipocytes which are centre of adiposity from where aberrant signals originate to initiate various abnormal biochemical outcomes (Fig 13). The struggle begins between physiological and non physiological forces and diseases set in when physiological processes are overwhelmed. The positive energy balance adversely affects almost all cells of the body but major affected ones are adipocytes, hepatocytes and beta-cells of Langerhans in pancreas. Obviously, these abnormalities make obese persons very prone to DMT-2 (Fig 14).

Beta-Cell Dysfunction

The failure to adapt long term demands of insulin due to IR and other factors leads to dysfunction of beta-cells.

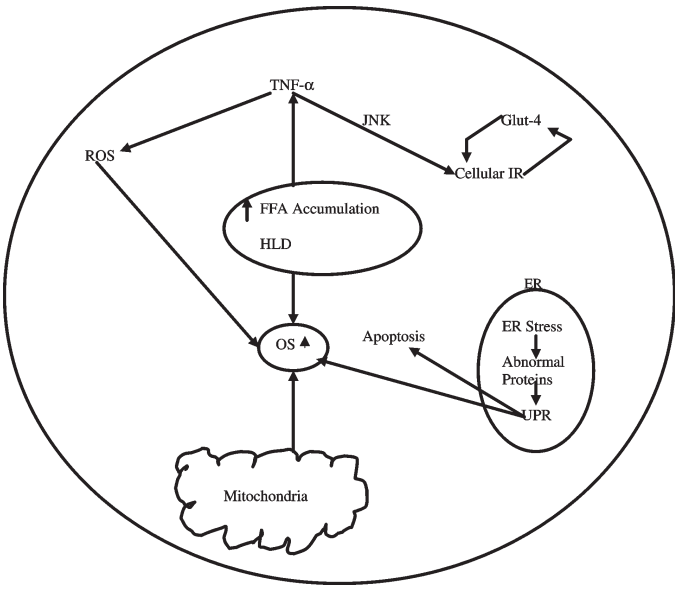

Fig 13 : Defects in adipocyte function
HLD-Hypertrophied Lipid Droplet; ER-Endoplasmic Reticulum; URP-Unfolded Protein Response

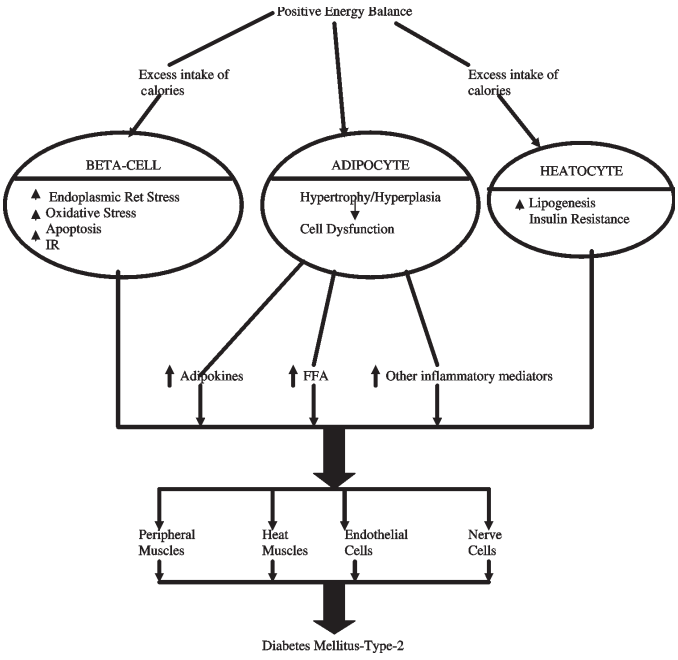

Fig 14 : Pertubations due to excess energy intake

Qualitatively it is seen as loss of normal oscillating and pulsatile pattern of insulin secretion. Quantitatively it is associated with progressive loss of beta-cell mass, degeneration of islets and deposition of protein amylin. OS in general and $O^{-\cdot}_2$, NO and H_2O_2 in particular have been demonstrated to injure beta-cells and their activity (15, 17). The beta-cells are relatively more susceptible to ROS and RNS injury as they have lower activity of antioxidant enzymes superoxide dismutase, catalase and glutathione peroxidase. Accompanied with it is lower activity of an important endogenous antioxidant thioredoxin. Initially the activity of antioxidant enzyme production increases to counter the increased FR activity but beta-cells have limited capacity to adapt. Interestingly, it has been demonstrated that generation of ROS in HIT-T15 cells transfected with human glucokinase gene caused a significant reduction in RNA and protein expression and N-acetyl-cysteine nearly nullified this effect. DMT-2 is known to be accompanied with raised levels of free fatty acids (FFF) in blood and tissues and several lines of evidences suggest that this raised level free fatty acids leads to low grade raised chronic OS which accelerates the beta-cell apoptosis through aggravated nitric oxide production. This has been supported by inhibition of NO production by antioxidants (Fig 15) (17).

Diabetic Complications

It is well known that several complications get associated in DMT-2 (103). Numerous clinical (5, 15, 42, 43, 54, 62, 73,104-107) and animal studies demonstrated that these are mainly due to chronic hyperglycemia intercalated by FR and OS (75-

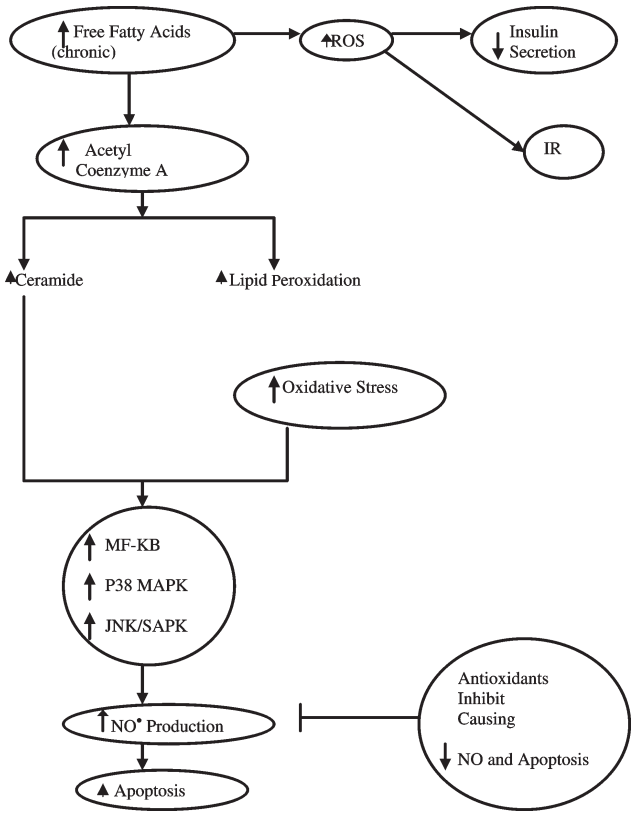

Fig 15 : Effect of chronic increase of free fatty acids on decreased insulin secretion and increased apoptosis in beta-cells of Langerhans

79,108). Usually these complications set after several years of hyperglycemic condition. DMT-2 has usually a long period of asymptomatic hyperglycemia before the detection of the disease. As such a good number of patients have already preexisting complications at the time of diagnosis. This is still more so in developing populations where health care is in much lower order of priority, and those patients from lower and middle socio-economic strata seek medical help only under compelling health conditions. These chronic complications can be divided into vascular and non-vascular complications. The vascular complications are subdivided into two categories viz. microvascular (neuropathy, retinopathy and nephropathy) and macro-vascular (coronary artery disease, peripheral arterial disease and cerebrovascular disease). The non-vascular complications include cataracts, glaucoma etc. OS has been implicated in all vascular complications and in some non-vascular complications especially cataract (14, 15, 16, 73). The glycemic control may prevent or delay the complications but in any case it does not guarantee for it. Diabetic complications are one set of conditions where FR injury is considered to be a definite etiologic factor in selective DMT-2 patients. The Figure 16 shows the different routes which can contribute to inflammed ROS, RNS and OS. In turn they participate in the development of complications. These are mostly related to vasculopathies and cataract. The proposed mechanism for their participation is that hyperglycemia causes mitochondrial dysfunction and over expression of many enzymes which causes an increased O^{-}_{2} production. This in turn enlarges ROS and RNS pool. These reactive molecules then intervene to cause pathological complications. The AGE and sorbitol routes are more prominent routes compared to protein kinase C and hexosamine pathway (17-19). The concentration of AGEs directly correlates with hyperglycemia. These compounds cause cross- linking of proteins which is more pronounced with long lived proteins such as collagen and extracellular matrix proteins, glomerular dysfunction, endothelial dysfunction, changes in composition and structure of extracellular matrix.

Nitric oxide synthesis is decreased which affects vascular tissue remodeling. Normally 2-5% of glucose is diverted to glucosamine pathway. It moves upstream in DMT-2. Similar trend is visible for sorbitol pathway. The pathways raise OS. All the changes taken together are reported to disturb redox homeostasis (inducing prooxidizing environment), increased cellular osmolality and diverse cellular functions (15, 16). Hexosamine pathway stimulation results in an increased production of amino sugars especially glucosamine which enhances glycosylation and proteoglycan synthesis. Several proteins including nitric oxide synthase are glycosylated resulting in altered properties. Further, gene expression for several proteins is also altered. Mitochondrial ROS aggravates the synthesis of diacyl glycerol (DAG) and protein kinase C (PKC) which have been shown to modify gene transcription, collagen, fibronectin, contractile proteins and extracellular matrix proteins in endothelial cells and neurons. Recently Aruoma et al (16) have critically assessed the contribution of FR in DMT-1 and DMT-2 complications. They have concluded that FR can induce DNA modifications such as mutations and complex DNA rearrangements. Chronic and severe diabetes in mothers can lead to genomic injury in fetus causing defective embryonic development. AGEs can accelerate vascular occlusion by quenching the vasodilatation agent nitric oxide. Their interaction with high affinity receptors located on monocytes and macrophages enhance and stimulate the FR formation, TNFα, Interleukin-1 and insulin like growth factor-1, which can proliferate endothelial, mesangial and smooth muscle cells thereby contributing to vascular complications. Retinopathy is associated with increased vascular permeability, vascular occlusion alongwith the growth of new blood vessels in the retina and on the posterior surface of the vitreous. ROS cause decreased retinal blood flow, increased vascular

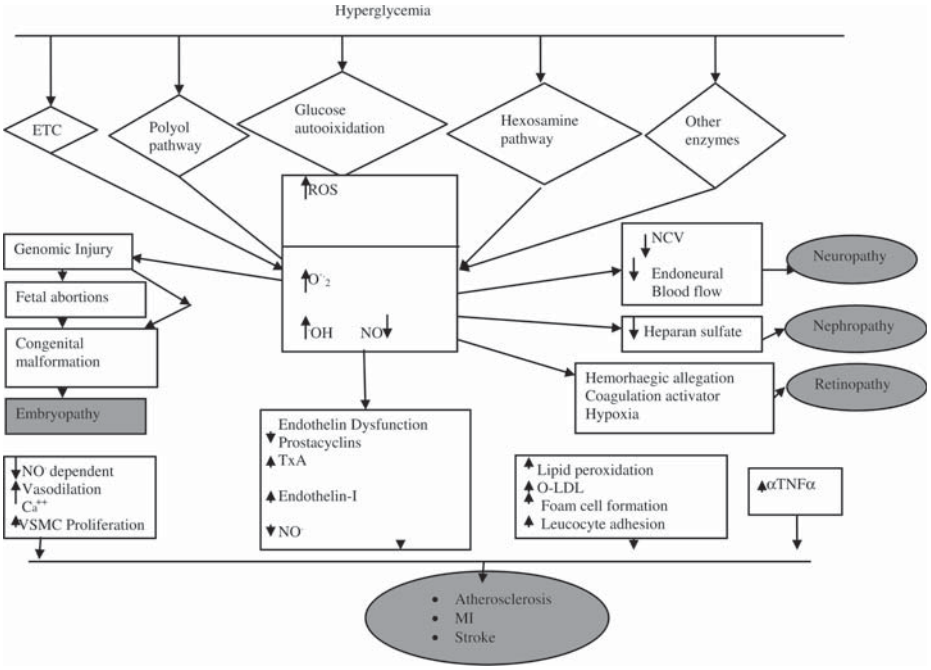

Fig 16 : Various complications in DMT-2 caused by hyperglycemia induced oxidative stress

permeability and disruption of blood-retinal barrier. Instead of treatment with pure antioxidant, management with medicinal and food plants has been advocated as plants are imbued with a diverse group of photochemicals, consisting of both antioxidants and non-antioxidants, which are beneficial in the management of DMT-2 and DMT-1 patients (16). Roberts and Sindhu (107) have asserted that metabolic syndrome is a collection of cardiometabolic risk factors which includes obesity, insulin resistance leading to DMT-2 and cardiovascular complications in which ROS are important participants. Nathan (97) believes that inflammation is major driver in the etiopathogenesis of DMT-2 and its complications and in many other chronic diseases and that ROS and RNS help to drive chronic inflammation. As a corollary, diabetic complications due to diverse mechanisms are major problems in all the populations where in ROS and RNS seem to be discernible players but surprisingly available antioxidants do not seem to confer any decisive benefit.

Antioxidant Therapy and Supplements

"Just few years ago, antioxidant pills were rising stars in the fight against heart disease but now they appear heading for the dustbin of history"

(Health & Nutrition, October 2001, P-63).

Only last month in Nature Ulrich Theopold (52) has appropriately said "Few concepts have been embraced by popular science as enthusiastically as the idea that the reactive oxygen species (ROS) are harmful and their levels should be controlled by including antioxidants in the diet or as supplements". He is therefore right to point out that this concept accepted so hurriedly without proper scientific verification was expected to change. It is therefore not surprising that it has gone through radical changes in last two decades. Initially antioxidants were projected as miracle species with an inherent quality just short of elixirs (109-116). Even now some scanty reports favour their liberal use (117). In second phase, with many disappointing clinical outcomes, the wisdom of calling them as miracle molecules was questioned (12-15, 18, 42, 46,118). In the recent phase, growing clinical and experimental evidence suggests that the excess intake of antioxidants may not only harmful effects but may cause increased mortality as well (119, 120).

Only nine years ago Pryor (115) stated that 50% cardiologists in USA took supplemental vitamin E and concluded that supplementation of vitamin E is extremely useful for health. Several studies published recently abrogate them. For example Lin et al (12) concluded that vitamin C, E and beta-carotene supplementation has no benefits in the primary prevention of total cancer or cancer mortality. Likewise there are similar conclusions for various diseases including diabetes mellitus (122). A step ahead, there are numerous clinical and experimental studies and Cochrane metaanylysis or other statistically designed conclusions which suggest harmful effects of antioxidants in various diseases (123-125).

Thus the results of clinical trials on DMT patients and for that matter in other diseases such as CVD cancer, aging etc can broadly be divided into four categories: I)beneficial effects II)no effects III)harmful effects and IV)increased mortality. A long drawn study of 23 yr follow up in a cohort in Finland consisting of 2285 men and 2019 women for 4 tocopherols, 4 tocotrienols, 6 carotenoids and ascorbic acid showed that dietary intake of these of antioxidants reduced the risk of DMT-2 (126). The flaw of this study is that it took only these antioxidants from the diet into consideration whereas the diet contains a large number of other ingredients which might be exerting beneficial effects, such as variability of the diet appears to be a telling confounding factor in this study. Another study from the same

country by Reunanen et al (127) reported that serum beta-carotene and α-tocopherol concentrations were associated with a reduced risk in diabetes. Again it may be pointed out that this association was not significant. Beckman et al (128) reported that administration of vitamin E and C in the doses of 800 IU/d and 1000 mg/d respectively for six months alleviated vasorelaxation in DMT-1 but not in DMT-2. Another A combination of vitamins E and C in the doses of 680 mg/d and 1250 mg/d improved renal function in DMT-2 patients. In yet another study Gaede et al (129) compared the multifactorial effects of intensive therapy with that of conventional treatment. The therapy consisted of antioxidants vitamin E (100 mg/d) ascorbic acid (250 mg/d), folic acid (400 mg/d) and chromium picolinate (100 mg/d). It reduced CVD effects by 50% in DMT-2 patients as compared to conventional therapy. Alpha-lipoic acid is one of versatile antioxidants though with variable effects in diseases. Some studies report its benefits in diabetes without complications and in neuropathy (110). In HOPE trial consisting of 1838 diabetics receiving vitamin E (400 IU/d) and 1816 receiving placebo, no effect of vitamin was observed after 4.5 yr of supplementation for heart failure, revascularization, nephropathy and total mortality (130). In a 15 year prospective study initially designed to investigate risk factors in diabetes and mortality in 41836 post-menopausal women in the age group 55-69 years, a secondary analysis was carried out to ascertain the effects of ascorbic acid in women suffering from DMT-2. Ironically the conclusion was that vitamin C supplement was associated with increased CVD deaths in DMT-2. Bjelawick et al (131) in a comprehensive analysis of the data of 105065 participants concluded that antioxidant alone or in combination increased the risk of all cause mortality as compared to placebo Homes and McCance (132) conducted a trial of ascorbic acid (1000 mg/d) and α-tocopherol (400 mg/d) for prevention of eclampsia. The primary end point was pre-eclampsia and secondary end point was birth weight of newborns ($\leq$ 2.5 kg). No effect was visible on the incidence of pre-eclampsia. However, birth weight of newborns was lower whose mothers were taking vitamin E supplements. There is yet another study to support it (133). Collectively these studies suggest that use of antioxidant for prevention of eclampsia is not warranted. On the contrary these antioxidants may have adverse effects. In their concluding remarks in a review Clarke and Burnett (123) state that antioxidant supplementation should be avoided unless deficiency exist, lest they may lead to undesirable effects. Lately in 2009, Women's Antioxidant Cardiovascular Study (WACS) reported the influence of nutrient antioxidants on the risk of DMT-2 in women with high risk of cardiovascular diseases (134). Among 6574 women, 895 cases developed DMT-2 in 9.2 year follow up. The supplementation of RRR-alpha-tocopherol acetate (600 IU. alternate day) slightly but non-significantly increased the risk of diabetes. Ascorbic acid (500 mg) slightly but non-significantly decreased the risk whereas beta-carotene had no effect. In their final conclusion, they said that neither of these nutrients had any positive or negative effect on the risk of the development of diabetes and that almost similar conclusion have been derived by Pocobelli et al (134) on supplementation of multivitamins, vitamin C and E in relation to mortality in cardiovascular disease and cancer. Likewise there are numerous reports claiming both favourable and adverse effects of antioxidant treatment and supplementation for an ideal health and for the treatment of human diseases.

Thus strikingly the developments in both therapeutic and nutritional circuits have punctuated with some success and some spectacular failures. Recent studies mostly favour that additional antioxidants should be given only when preexisting antioxidant deficiency is present and that long term use may result in adverse outcomes due to alteration in metabolic points or changes in redox homeostasis. This is true for diabetes also. In October issue of NATURE 2009, Owusu-Ansah and Banerjee (53) have demonstrated the useful effects of moderately raised OS in fruit fly and have said in their closing remarks "The finding that ROS levels are moderately high in normal Drosophila haematopoietic progenitors and mammalian common myeloid progenitors raises the possibility that wanton over dose of antioxidant products may infact inhibit the formation of the cells participating in the innate immune response". We concur with them. Two years earlier we have already said the same thing in an editorial (13). Very recently in an editorial Singh and Sharma (135) have pointed out strength and weaknesses of antioxidants. Unless explicit attention is paid to ascertain whether the effect of ROS, RNS and OS in a disease is a priori (from effect to cause) or a posteriori (from cause to effect), selection of antioxidants will continue to faulter.

CONCLUSION

In summary, the glucose homeostasis is a cusply coordinated process and is operated by several parallel, serial and cross switches. Indeed, the hormone insulin is the most powerful molecule to act as a central director through an army and arsenal of intracellular and extracellular molecules known as "Insulin Signaling Cascade". Ironically our knowledge in this regard is still incomplete. Influence of ROS, RNS and antioxidants has been under scanner on this cascade in triggering diabetes. Evidence is mounting to suggest : I)ROS and RNS are important regulators of glucose homeostasis in blood and tissues and this deregulation may trigger DMT-2

and may set additional complications in due course of time. II) the FR could be causative factors is the genesis of IR. III) Redox perturbations in mitochondria consequent to altered ATP generation in electron transport chain results in enhanced leakage of electrons to form more superoxide anion. IV) metabolic changes in cytosol consequent to changes in enzyme activities of NADPH oxidase, xanthine oxidase uncoupled nitric oxide synthase and many others increase the generation of reactive species. Both of these activities may participate in the pathogenesis of DMT-2. V) many environmental factors overwhelmingly tilt the redox homeostasis toward proxidizing conditions which affect the insulin signaling cascade and genetic disposition to diabetes and lastly. VI) the reactive species may promote diabetes risk by provoking genetic factors. Antioxidants are undoubtedly essential spokes of human life but their excess intake is undesirable. Further our studies on several series of diabetic patients and those of others indicate that raised OS is not an inevitable phenomenon in diabetes.

Thus, despite all the loaded evidence for the involvement of reactive species in the diabetes, the debate continues on three points: a) is it selective in patients or present in all patients but not detectable by available methods, b) is it facultative, that is, it is capable of causing disease but does not necessarily do so in all patients and c) is it obligatory, that is, it universally participates in the genesis of diabetes. As the evidence stands today, the participation of ROS and RNS is selective and facultative. Antioxidants have so for not received putative pat in the medicine though FR involvement has lately been given recognition. The crux of the lesson is that controversies should not deter and discourage us rather infuse determination in us to carry the work to final destination by scrupulously veracious precision work. We would only like to post a caution “Those who fail to read the history are destined to suffer from repetition of the mistakes”. We must therefore tread carefully with exactitude in future.

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