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Diabetic Complications, Hyperglycemia & Free Radicals

by

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Abbreviations: (AGE) Advanced Glycation Endproducts, (ALA) α-Lipoic Acid, (DETAPAC) Diethylenetriamine-pentaacetic acid, (DHA) Dehydroascorbic Acid, (GLUT) Glucose Transporter, (GSH) Glutathione, (NIDDM) Non-insulin Dependent Diabetes Mellitus, (ROS) Reactive Oxygen Species, (SOD) Superoxide Dismutase,

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ABSTRACT: Diabetes mellitus, which literally means “sweet excessive urine”, is an extremely common disease in the United States. It is characterized by either a deficiency in insulin, or resistance to insulin. Diabetic patients also suffer from a wide variety of complications due to their disease, such as artherosclerosis, retinopathy, poor circulation, and liver and kidney problems. Diabetes also seems to be accompanied by a shortage of antioxidants and an increase in free radicals, the end result being oxidative stress. Research is being done to determine if the incurring oxidative stress is just another complication or if it may be a cause, in part or in full, of some of the diabetic complications. This paper will discuss the role of free radicals in the pathology of diabetes and its complications.

INTRODUCTION

There are two classes of diabetes mellitus, Type I and Type II, with type II compromising over 80% of the clinical cases [23]. Type I (also called juvenile or insulin-dependent diabetes mellitus) generally develops when patients are in their adolescence and is characterized by the destruction of the β cells in the islets of Langerhans, which are responsible for the production of insulin. Type II diabetes (non-insulin dependent diabetes mellitus) generally develops later in life and is caused by insulin resistance. The reasons behind this resistance to insulin vary widely, often involving environmental causes and genetics. Obesity and family history are prime risk factors for the development of NIDDM. Type I diabetes is treated with insulin supplementation, while type II can often be controlled with just diet and exercise. If these measures are insufficient, there are also a multitude of oral hypoglycemic agents available for the treatment of this disease. Insulin is used only when the previous measures fail.

Diabetic patients have an increased level of blood glucose, but many patients also have secondary effects from the diabetes, such as poor circulation, ischemic brain injury, platelet adhesion and aggregation, excess free radicals, shortage of antioxidants, heart disease, cataracts and liver and kidney problems. Most of these are caused, at least in part, by too much glucose. Excess glucose, hyperglycemia, can be toxic to cells in several ways, two of which are the formation of advanced glycation end products (AGE) and free radicals such as $O_2^{\bullet -}$, $^{\bullet}OH$. Both types of products can contribute to diabetic complications. Excessive free radicals can come from several pathways;

ischemia, hyperglycemia, increased mitochondria leak, catecholamine ocidation and leukocytes [13]. Diabetic subjects have been shown to have increased levels of superoxide and hydrogen peroxide. Changes in antioxidant enzymes and small antioxidant molecules have also been documented.

HYPERGLYCEMIA AND FREE RADICALS

Hyperglycemia can increase the levels of free radicals through protein glycation, autoxidation glycation, protein kinase and an increase in the polyol pathway. Autoxidation of glucose is the process by which it enolizes. This process entails the

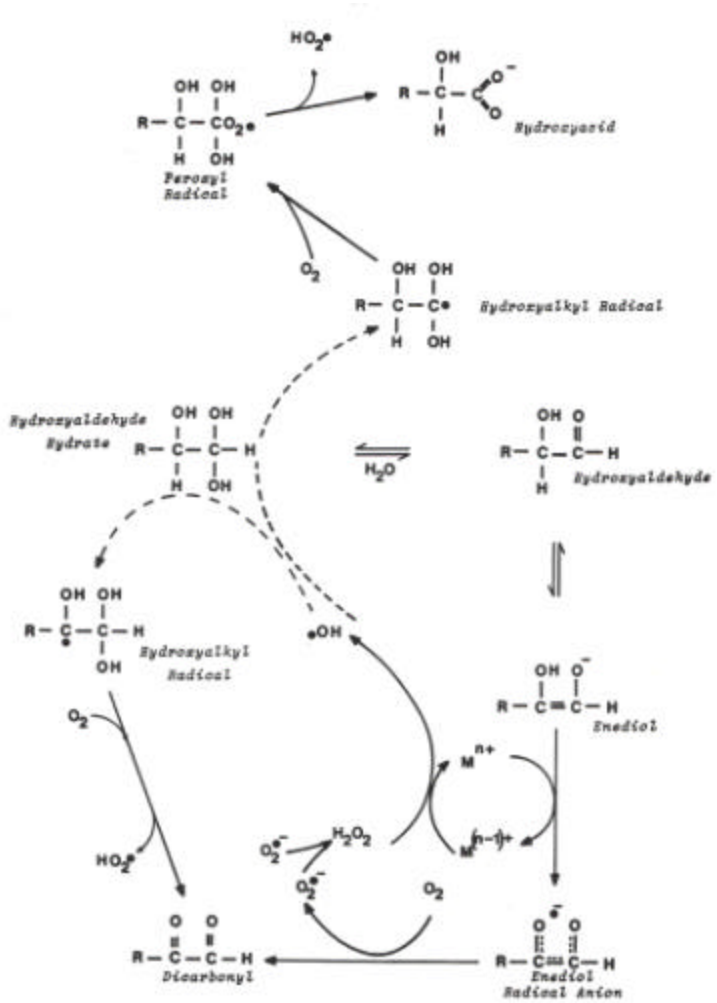

Figure 1. The process of autoxidation of a monosaccahride, showing how free radicals are produced when excess glucose is present [26].

reduction of oxygen, producing oxidizing intermediates, such as $O_2\bullet^-$, $\bullet OH$ and H_2O_2, and α-ketoaldeydes[9]. See Figure 1. These molecules can damage important biomolecules such as DNA, proteins and lipids. The oxidizing intermediates formed by autoxidation is proposed to be a cause for some of the structural damage seen in diabetes. This reaction is often catalyzed by transition metals, and even with the catalyst, the reaction is very slow. These ketoaldehyde products may attach to proteins, in a process is called labile glycation. Protein fragmentation and labile glycation due to glucose autoxidation can be reduced by the use of a chelating agent, like DETAPAC [8].

Glucose can also undergo glycation directly, where the glucose molecule covalently bonds to a protein to form a Schiff base. These molecules can then undergo rearrangement to form an Amadori adduct. Amadori adducts can then decompose to form deoxyglucones, which are considerably more reactive than the sugar they derived

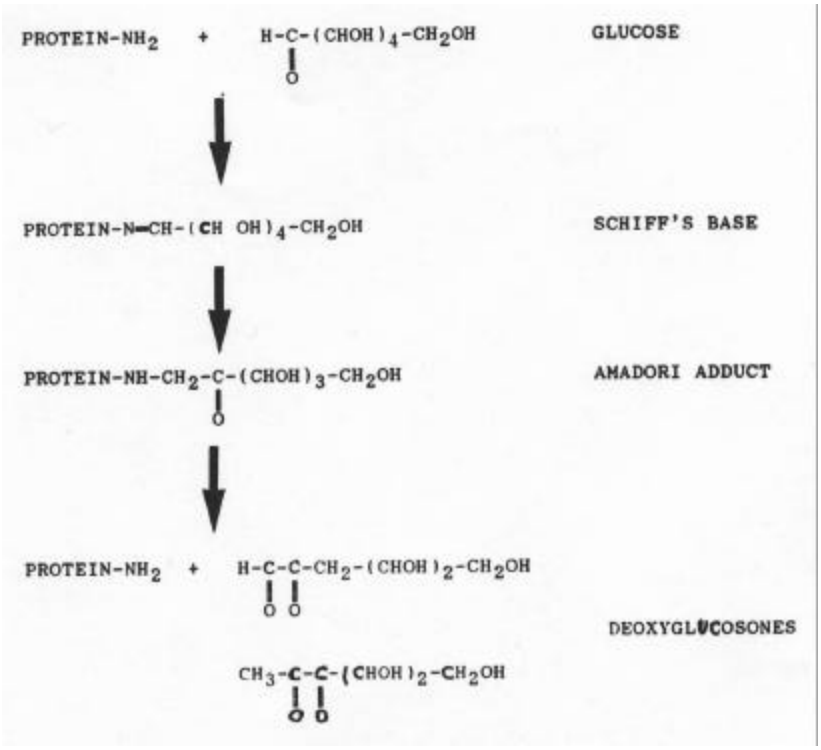

Figure 2. Glycation process and subsequent degradation of glycation products [26].

from (Figure 2). These more reactive ketoaldeyhdes may go on to react with other proteins to form **A**dvanced **G**lycation **E**ndproducts (AGE) or Maillard products [26]. The Maillard products lead to the "browning" of the protein, the protein also becomes fluorescent and crosslinked. Glycation is a reversible process. When glycation follows autoxidation, also called glycoxidation, the products tend to be more permanent modifications such as protein crosslinking. Hemoglobin glycation is commonly used clinically to monitor the blood sugar level over several weeks. The amount of hemoglobin glycation can help doctors and patients monitor the glycemic control, or lack of.

An increase in the concentration of glucose contributes to an enhanced activity of the two enzymes used in the polyol pathway, aldose reductase and sorbitol dehydrogenase. With the increased activity of these two enzymes, the concentration of both sorbitol and fructose increase. This increased activity also causes the NADPH:$NADP^+$ ratio to decrease and the NADH: NAD^+ ratio to increase [7]. The change in these ratios can cause changes throughout various systems in the cell. The increase in the NADH:NAD+ ratio, also called hyperglycemic pseudohypoxia, may cause an increase in free radical production which may lead to ischemia. It may also produce a reduction in glycolysis, which results in reduced pyruvate levels [12]. The reduction in the amount of NADPH may cause an inhibition in enzymes which are NADPH-dependent and lead to a shortage of the NADPH available for the many pathways it is involved in.

Glucose, once phosphorylated to glucose-6-phospate, is metabolized through two main processes in the cell, glycolysis and the pentose phosphate pathway. Glycolysis results in the production of pyruvate, which then goes on to react in the tricarboxylic acid cycle, among others and is also known to scavenge H_2O_2 and other hydroperoxides. The pentose phospate pathway produces NADPH, which is the primary source of reducing equivalents for the glutathione reductase system, among many other oxidizing species. So not only is glucose a primary energy source, it is also a means of removing toxic H_2O_2 and hydroperoxides from cells.

ANTIOXIDANTS

Vitamin E is an antioxidant that resides in the lipid bilayer of the cell membrane. Its purpose is to stop the chain reaction begun by lipid radicals. Vitamin C then reacts with the α-tocpherol radical, thereby removing the radical from the lipid bilayer and moving it into the cytosol where it can be dealt with by resident enzymes (Figure 2).

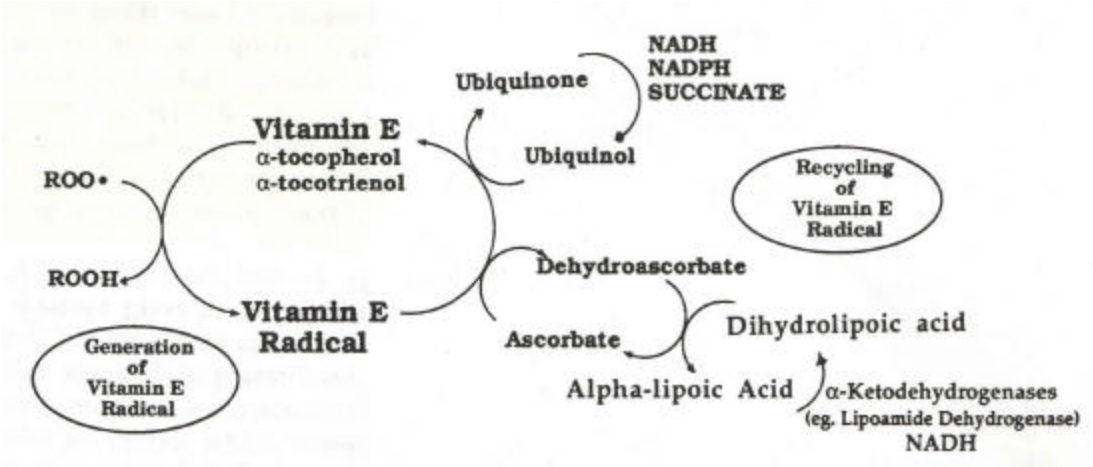

Figure 3. Recycling of vitamin E [16].

$$2 \text{ vit } E^{\bullet} + \text{vit C} \rightarrow 2 \text{ vit E} + \text{DHAA}$$ **Equation 1 [2]**

GSH can also regenerate vitamin E, forming GSSG which can then be reduced by glutathione reductase back to GSH.

$$2 \text{ vit } E^{\bullet} + 2 \text{ GSH} \rightarrow 2 \text{ vit E} + \text{GSSG} \qquad \textbf{Equation 2 [2]}$$

$$\text{NADPH} + \text{GSSG} \rightarrow \text{NADP}^{+} + 2 \text{ GSH (glutathione reductase)}$$

DHLA is able to regenerate both vitamin C and GSH without the use of enzymes. This allows the indirect regeneration of vitamin E, helping to prevent lipid peroxidation.

$$\text{DHLA} + \text{GSSG} \rightarrow \alpha\text{-LA} + 2 \text{ GSH} \qquad \textbf{Equation 3 [2]}$$

$$\text{DHLA} + \text{DHAA} \rightarrow \alpha\text{-LA} + \text{vit C}$$

GSH is also able to regenerate vitamin C, but DHLA shows greater reactivity towards it (Table IV), so at specific concentrations, DHLA will regenerate vitamin C preferentially.

Reducing Agent	k (M / min)
GSH	32.8
DHLA	875

Table I. Regeneration rate constants for vitamin C. [16]

A high level of glucose is thought to hamper the activity of antioxidants, such as superoxide dismutase. A study was done in which varying levels of glucose were mixed with Cu,Zn-SOD. In this study, a decrease in the activity of the enzyme was found at higher levels of glucose [8]. Various antioxidants have been studied in different tissue types of normal and diabetic animals. Vitamin C and vitamin E, both essential antioxidants, have been reported to be decreased in chemically-induced diabetes [25]. It has also been found that SOD and glutathione peroxidase are both reduced in diabetic individuals. CuZn-SOD levels increase slightly after insulin treatment [13].

ASCORBATE & GLUCOSE TRANSPORTERS

Ascorbate is transported into cells *via* two mechanisms. Ascorbate can be brought into the cell directly through an undetermined Na^+-dependent transporter. Ascorbate can also be oxidized outside the cell, allowing it to be transported by several of the glucose transporters into the cell, where it is reduced to ascorbate again. DHA has a structure very similar to that of glucose (Figure 3), which allows several of the GLUT family to act as DHA transporters.

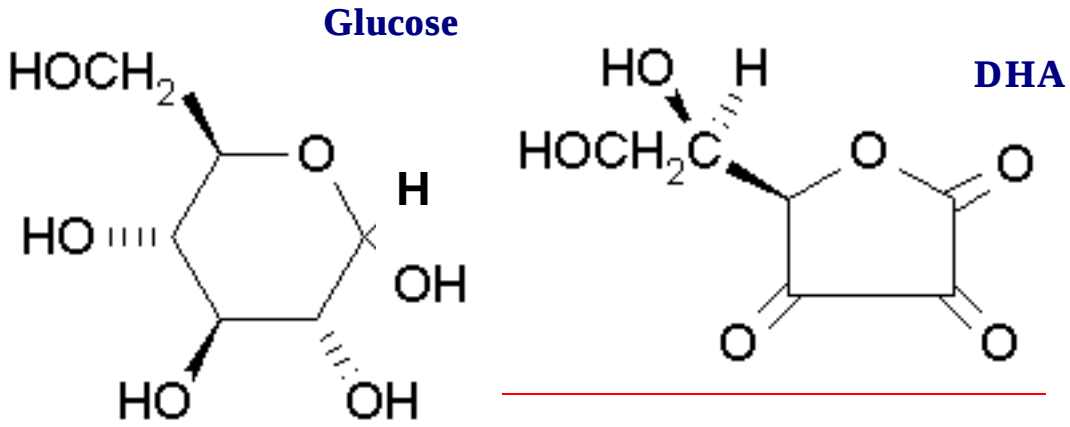

Figure 4. The structure of glucose and DHA [27].

GLUT1, 3 and 4 are able to move DHA into the cell (Table II). GLUT4 is only able to transport appreciable amounts of DHA after insulin stimulation, which stimulates the GLUT4-containing vesicles to transport GLUT4 to the membrane.

Table II. K_m for DHA transport by GLUT family proteins [20,21].

Protein	K_m
GLUT1	1.1 mM
GLUT3	1.7 mM
GLUT4 (+ insulin)	0.98 mM

DHA uptake and its subsequent reduction can account for an increase in ascorbate accumulation of 5-20 fold within of minutes of insulin stimulation [21]. This process, called ascorbate recycling, allows the cell to respond quickly to oxidative stress.

Diabetic patients tend to have a condition that has been termed “micro-scurvy”, meaning that the cells have a vitamin C deficiency. The amount of vitamin C entering the cells of diabetic patients through the GLUT proteins can be reduced in two different ways. If there is decreased GLUT4 translocation to the membrane, less DHA will be able to enter the cell *via* the transporter. Vitamin C must also compete with glucose for entrance into the cell via the GLUT family transporters. If the flux of glucose into the cells is increased, as it is in diabetes, then less vitamin C will be able to enter the cells. It has not been conclusively proven whether the ascorbate deficiency found in diabetic patients is the result of reduced concentrations in the cell or an increased requirement for ascorbate.

OXIDATIVE STRESS AND GLUCOSE TRANSPORTERS

Prolonged oxidative stress has a significant effect on GLUT1 and GLUT4. GLUT1 expression is increased when the cell is exposed to low-grade oxidative stress for an extended period of time, while GLUT4 is reduced. An 18 hour exposure of 3T3L1 cells to H_2O_2 (produced by glucose oxidase) resulted in an approximate 3.5-fold increase in GLUT1 protein and mRNA and a simultaneous 45% reduction of GLUT4. This experiment showed a reduction in insulin-stimulated glucose uptake, glycogenesis and lipogenesis. Oxidative stress also impairs GLUT4 translocation, but has no effect

on GLUT1 translocation. The changes in GLUT1 and GLUT4 expression and activity can have a substantial effect on the cell's ability to uptake glucose, which in turn effects a multitude of metabolic processes.

DIABETIC COMPLICATIONS AND FREE RADICALS

Hyperglycemia can produce a wide variety of secondary diabetic complications. Oxidative stress plays an integral role in the development of complications due to excess glucose.

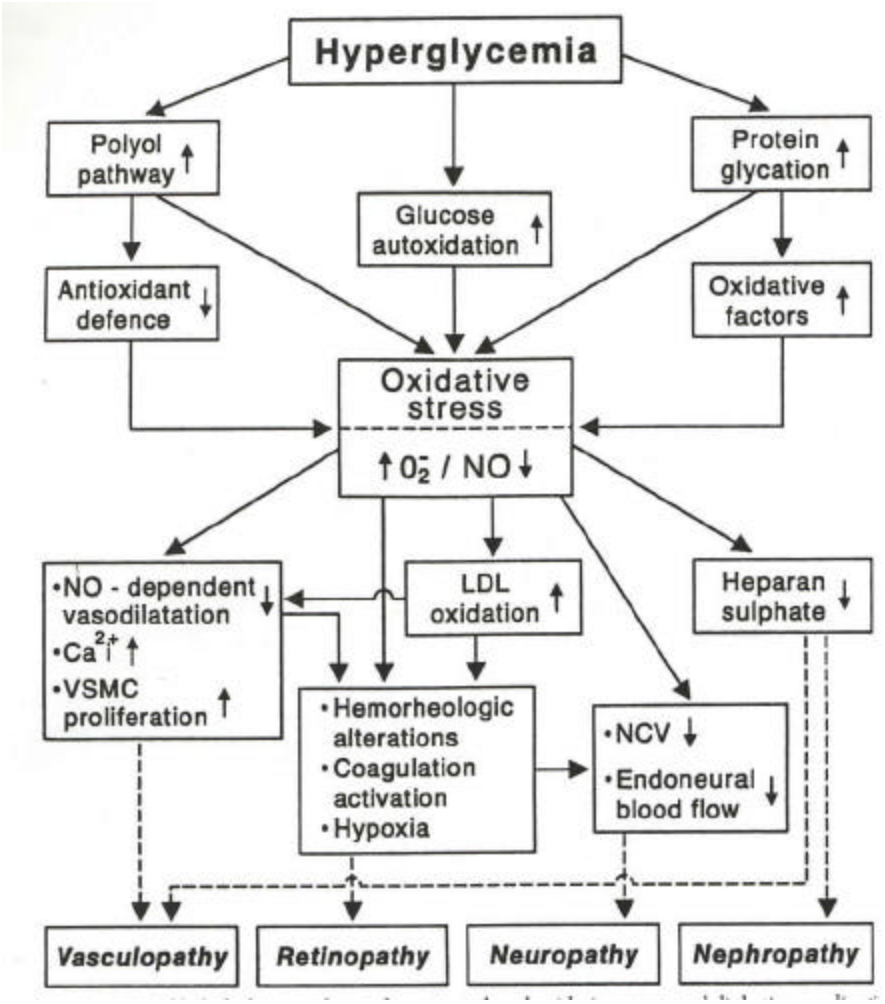

Figure 6. Excessive glucose can cause multiple secondary complications through a variety of pathways, which are appear to lead to oxidative stress[7].

Diabetic patients have been shown to have platelets with increased adhesiveness and aggregation, increased concentrations of thromboxane A_2, platelet factor 4 and β-thromboglobulin [17]. Increase in platelet aggregation can cause a variety of effects: vasoconstriction, anoxia, ROS, atherosclerotic plaques, retinopathy, nephropathy and CV disease. Figure 6 shows how hyperglycemia can be related to oxidative stress and further complications.

Gliclazide is a sulphonylurea, used to maintain glycemic control. It has been shown that gliclazide appears to have an effect on some of the diabetic complications. Gliclazide is able to reduce the increase in platelet adhesiveness and aggregation, it has also been shown to reduce the release of platelet factors. Gliclazide causes a reduction in anoxia, reperfusion damage and ROS, while increasing fibrinolysis and basement membrane thickness. Figure 6 details some of the haemobiological actions of gliclazide. The net effect of these actions is a reduction in the risk of atherosclerosis.

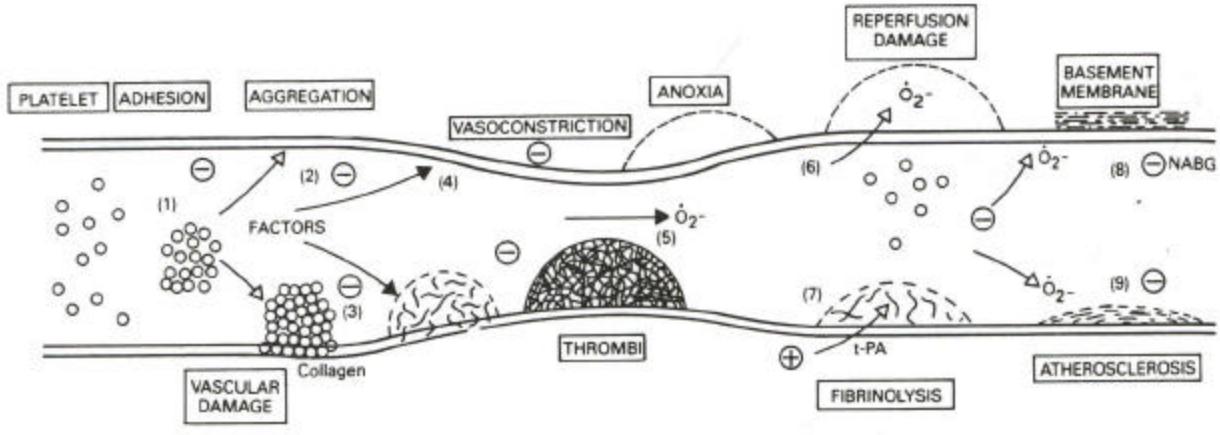

Figure 7. Hemobiological effects of gliclazide. Gliclazide causes a reduction in platelet adhesion and platelet factors, which in turn decrease platelet aggregation, vasoconstriction and vascular permeability. This allows oxygen flow to increase, reducing anoxia and reperfusion damage caused by free radicals. It also stimulates tissue plasminogen activator, which increases fibrinolysis and basement membrane thickness [4]. (+) increases, (-) decreases

Another drug known to have beneficial effect on the diabetic complication is ALA. ALA is an antioxdant that is currently approved for diabetic neuropathy in Germany. A 50% reduction in nerve blood flow has been found in streptozotocin experimental diabetic animals. This reduction reverted to normal after one month of treatment with α-lipoic acid (100 mg / kg). The increase in nerve blood flow after ALA treatment appears to be dose-dependent (Figure 8). While this effect of ALA on experimental diabetic rats is impressive, it is yet to be seen if ALA will have such a substantial result on human diabetes.

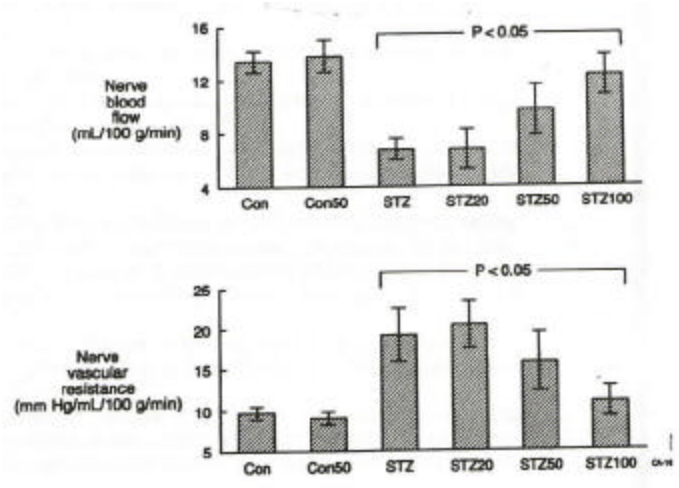

Figure 8. The nerve blood flow and vascular resistance are shown for control animals, streptozotocin treated animals, which have had different doses of ALA over a month period (0-100 mg / kg) [13].

FUTURE DIRECTIONS

There are two primary ways of developing diabetic animals. Treating them with streptozotocin or alloxan. Both of these compounds act, at least in part, through reactive oxygen species. Alloxan and streptozotocin attack the pancreatic β cells, which are responsible for the production of insulin. This causes the induction of a type I diabetic state, i am unaware of an animal model for type II diabetes. Though these animals are an extraordinarily valuable research tool in diabetic study, it is impossible to determine the correlation between these animals and human diabetic subjects. Antioxidant studies should be done with these animals to further elucidate the role of ROS and antioxidants in diabetes and its complications.

Since adipocytes are an important cell type in type II diabetes, they can be used to further elucidate the molecular effects of antioxidants on the cell processes. Adipocytes are produced by differentiating 3T3 L1 fibroblast cells. The cells can be transfected with SOD or catalase or incubated in small molecule antioxidants to increase the antioxidant defenses level. The glucose uptake would be measured and compared between the different antioxidants. Anti-sense mRNA could be used to block some of the natural antioxidant enzymes from being produced, causing an artificial shortage of certain enzymes. This would allow one to determine if another antioxidant can substitute for the “knocked-out” enzyme.

Two of the more important antioxidant enzymes, Mn SOD and CuZn SOD, are both found to be decreased in diabetic individuals. A lot of information could be

obtained by increasing the SOD levels artificially. This is extremely difficult to do *in vivo*, since SOD is a fairly large enzyme that is unable to cross plasma membranes. Because of this, the SOD is unable to make it into the cell where it can work on reducing superoxide. In cell culture, liposomal SOD can be used to bypass this problem. The liposomes allow the SOD to pass through the lipid bilayer of the plasma membrane. With further study, it may be possible to adapt liposomes for use in whole animals, but currently this is not acheivable in a consistent manner. Another possible method of increasing the removal superoxide is the use of SOD mimetics. SOD mimetics have superoxide scavenging ability, but are smaller and are able to gain access to the interior of the cell.

Cataracts are a major complication with diabetes. It is thought that a major culprit in cataract formation is oxidative stress. To test this theory, streptozocin or alloxan induced diabetic rats could be feed a diet containing varying combinations and levels of antioxidants. Since some antioxidants work better as a team (ex., vitamin C and vitamin E) it would be best to mix and match different antioxidants to see if there are synergistic effects between the antioxidants. Some antioxidants to be used would include vitamin C, vitamin E, ALA, GSH, among others. The formation of cataracts would then need to be monitored on a regular basis and in a reproducible manner.

A similar study could be done to test the impact different combinations of antioxidants would have on the overall animal.

I think it would be interesting to see an epidemilogical study of antioxidants and diabetes done. A wide subject base of diabetic individuals would receive either a

specific combination of antioxidants or placebos. Antioxidants to be used, such as vitamin C and E, are currently available as dietary supplements and several are routinely recommended by physicians for diabetic patients. It would be a double blind, multi-center study that would have to be followed for a long period. Antioxidant plasma levels, blood glucose control, lipid peroxide levels, TBARS and other oxidation markers could be measured regularly to allow interpretation of the study. α-Lipoic acid is approved for the use of diabetic neuropathy in Germany. A clinical study should be done in the United States to explore the dosage and efficacy of prescribing it as a preventive agent here. The clinical study on ALA should also be a double-blind, multi-centered study in order to achieve the most meaningful results.

CONCLUSION

I believe the evidence, though circumstantial in many ways, points to a distinct connection between free radicals and diabetic complications. An increase in free radical concentration has been shown for diabetic individuals. The increase in radicals can be caused by a variety of different factors, the most important of these being hyperglycemia. Accompanying this radical production is a decrease in many of the antioxidant defenses of the cell. Antioxidant enzymes, such as SOD and catalase, are generally decreased, as are several small antioxidant molecules like vitamin C and vitamin E. This decrease in antioxidants and increase in free radicals indicates a potentially dangerous situation for the cells and the specimen as a whole.

REFERENCES

1) Baynes JW. (1990) Role of oxidative stress in development of complications in diabetes. *Diabetes.* **40**:405-411.

2) Biewenga GP, Haenen GRMM, Bast A. (1997) The pharmacology of the antioxidant lipoic acid. *Gen. Pharmac*. **29**:315-331.

3) Borcea V, Nourooz-Zadeh J, Wolff SP, Klevesath M, Hofmann M, Urich H, Wahl P, Ziegler R, Tritschler H, Halliwell B, Nawroth PP. (1999) α-Lipoic acid decreases oxidative stress even in diabetic patients with poor glycemic control and albuminuria. *Free Rad Biol Med.* **22**:1495-1500.

4) Campbell DB, Lavielle R, Nathan C. (1991) The mode of action and clinical pharmacology of gliclazide: a review. *Diab Res Clin Prac*. **14**:S21-S36.

5) Ceriello A, Morocutti A, Mercuri F, Quagliaro L, Moro M, Damante G, Viberti GC. (2000) Defective intracellular antioxidant enzyme production in type I diabetic patients with nephropathy. *Diabetes.* **49**:2170-2177.

6) Fu M, Wells-Knecht KJ, Blackledge JA, Lyons TJ, Thorpe SR, Baynes JW. (1994) Glycation, glycoxidation, and cross-linking of collagen by glucose: Kinetics, mechanisms, and inhibition of late stages of the Maillard reaction. *Diabetes.* **43**:676-683.

7) Giugliano D, Paolisso G, Ceriello A. (1996) Oxidative stress and diabetic vascular complications. *Diabetes Care.* **19**:257-263.

8) Hunt JV, Dean RT, Wolff SP. (1988) Hydroxyl radical production and autoxidative glycosylation. *Biochem J.* **256**:205-212.

9) Hunt JV, Smith CCT, Wolff SP. (1990) Autoxidative glycosylation and possible involvement of peroxides and free radicals in LDL modification by glucose. *Diabetes* **39**:1420-1424.

10) Ido Y, Kilo C, Williamson JR. (1997) Cytosolic NADH / NAD+, free radicals, and vascular dysfunction in early diabetes mellitus. *Diabetologia.* **40**:S115-S117.

11) Kozlovsky N, Rudich A, Potashnik R, Ebina Y, Murakami T, Bashan N. (1997) Transcriptional activation of the GLUT1 gene in response to oxidative stress in L6 myotubes. *J Biol Chem.* **272**:33367-33372.

12) Lee AYW, Chung SSM. (1999) Contributions of polyol pathway to oxidative stress in diabetic cataract. *FASEB J.* **13**:23-30.

13) Low PA, Nickander KK, Tritschler HJ. (1997) The roles of oxidative stress and antioxidant treatment in experimental diabetic neuropathy. *Diabetes.* **46**:S38-S41.

14) Mohamed AK, Bierhaus A. (1999) The role of oxidative stress and NfκB activation in late diabetic complications. *Biofactors.* **10**:157-168.

15) Oberley LW. (1988) Free radicals and diabetes. *Free Rad Biol Med.* **5**:113-124.

16) Packer L. (1994) Antioxidant properties of lipoic acid and its therapeutic effects in preventation of diabetes complications and cataracts. *Ann. N.Y. Acad. Sci.* **738**:257-264.

17) Palmer KJ, Brogden RN. (1993) Gliclazide: An update of its pharmacological properties and therapeutic efficacy in non-insulin-dependent diabetes mellitus. *Drugs.* **46**:92-125.

18) Rösen P, Du X, Tschöpe D. (1998) Role of oxygen derived radicals for vascular dysfunction in the diabetic heart: Prevention by α-tocopherol? *Mol Cell Biochem.* **188**:103-111.

19) Rudich A, Tirosh A, Potashnik R, Hemi R, Kanety H, Bashan N. (1998) Prolonged oxidative stress impairs insulin-induced GLUT4 translocation in 3T3-L1 adipocytes. *Diabetes.* **47**:1562-1569.

20) Rumsey SC, Kwon O, Xu GW, Burant CF, Simpson I, Levine M. (1997) Glucose transporter isoform GLUT1 and GLUT3 transport dehydroascorbic acid. *J Biol Chem*. **272**:18982-18989.

21) Rumsey SC, Daruwala R, Al-Hasani H, Zarnowski MJ, Simpson IA, Levine M. (2000) Dehydroascorbic acid transport by GLUT4 in *Xenopus* oocytes and isolated rat adipocytes. *J Biol Chem*. **275**:28246-28253.

22) Tesfamariam B. (1994) Free radicals in diabetic endothelial cell dysfunction. *Free Rad Biol Med.* **16**:383-391.

23) Thompson KH, Godin DB. (1995) Micronutrients and antioxidants in the progression of diabetes. *Nutrition Research.* **15**:1377-1410.

24) Wells-Knecht KJ, Zyzak DV, Litchfield JE, Thorpe SR, Baynes JW. (1995) Mechanism of autoxidative glycosylation: Identification of glyoxal and arabinose as intermediates in the autoxidative modification of proteins by glucose. *Biochem*. **34**:3702-3709.

25) Wolff SP. (1993) Diabetes mellitus and free radicals: Free radicals, transition metals and oxidative stress in the aetiology of diabetes mellitus and complications. *British Medical Bulletin*. **49**:642-652.

26) Wolff SP, Jiang ZY, Hunt JV. (1991) Protein glycation and oxidative stress in diabetes mellitus and ageing. *Free Radical Biology & Medicine.* **10**:339-352.

27) http://www.sigma-aldrich.com/