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Possible protection of Vitamin E and Alpha-lipoic acid against early changes in alloxan diabetic rats.

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Abstract

Diabetes mellitus is a chronic syndrome affecting carbohydrate, fat, protein and nucleic acid metabolism. The current study was undertaken to elucidate the possible role of vitamin E and alpha lipoic acid in combination as an antioxidant and a biological membrane stabilizer in the protection against early complication of diabetes. Administration of alloxan (125 mg/kg wt, i.p.) to rats resulted in hyperglycemia, hyperinsulinemia, hypercholesterolemia, hypertriglyceridemia, hyperlipidemia, increase in plasma levels of urea, blood urea nitrogen (BUN), creatinine, uric acid as well as pancreatic thiobarbituric acid reactive substances (TBARS) and glutathione (GSH) content of both liver and retina. These changes were accompanied with significant decrease in plasma total protein, tumor necrosis factor α (TNFα), hepatic catalase activity (CAT), and TBARS level of both liver and retina as compared to control group. However, plasma levels of calcium ions (Ca^{+2}) and nitric oxide (NO) as well as pancreatic GSH content were not changed. On the other hand, the daily treatment of the diabetic rats with antioxidant mixture attained a reduction in plasma levels of glucose, cholesterol, triglycerides, total lipids, urea, BUN, creatinine, uric acid, TNFα, pancreatic TBARS level as well as GSH content of both liver and retina. In contrast, the daily treatment caused an increase in plasma levels of insulin, total proteins, hepatic CAT activity and pancreatic GSH content as compared to diabetic rats. However, plasma levels of Ca^{+2} and NO as well as TBARS content of both liver and retina were not affected. In conclusion, it is obvious from the present study results that early stage (two weeks) of diabetes induce deteriorate changes in carbohydrate, lipid, protein and nucleic acid metabolism accompanied with increasing of oxidative stress in pancreas as compared to both of liver and retina. Moreover, the data of present study indicated the effective role of vitamin E and alpha lipoic acid combination in combating the oxidative stress via its improvement to metabolism of carbohydrates, lipids, proteins and nucleic acids in addition to its free radical-scavenging and antioxidant properties.

Introduction

Diabetes mellitus is widely recognized as one of the leading causes of death in the world (Devendra *et al*., 2004). It is defined as a state in which homeostasis of carbohydrate and lipid metabolism is improperly regulated, resulting in elevated blood glucose levels (Ramkumar *et al*., 2007).

The presence of high glucose levels *in vivo* can alter a number of biochemical processes (Hopfner and Gopalakrishnan, 1999). This in turn may affect the function of different organs such as liver, pancreas, and retina (Ramkumar *et al*., 2007).

Numerous studies have shown that there is an increase in production and release of free radicals with diabetes and reported that they play a role in diabetic complications through glucose autoxidation and protein glycation (Wolff & Dean, 1987 and Hunt *et al*., 1990) as well as decreasing the activity of enzymic and nonenzymic antioxidant (Godin *et al*., 1988 and Asayama *et al*., 1989). High levels of free radicals cause damage to cellular proteins, membrane lipids, and nucleic acids resulting in eventually cell death (Maritim *et al*., 2003).

Currently-available drug regimens for management of diabetes mellitus have certain drawbacks such as vascular complications and hepatotoxicity (Ramkumar *et al*., 2007). Therefore, there is a need for safer and more effective antidiabetic drugs (Shu, 1998; Grover *et al*., 2002 and Kaleem *et al*., 2006). Antioxidants have been used extensively in experimental diabetes to

reduce or reverse the effects of free radicals.

The present study was carried out in order to estimate the effects of diabetes during early stage (two weeks) on the oxidative stress and antioxidant status of the alloxan diabetic rats. Also, this investigation has been undertaken to determine the mechanism of action of vitamin E and alpha-lipoic acid in combination to delay or prevent the onset or the progression of diabetes and their complications as well as its effect on the antioxidant status.

Materials and methods

Chemicals

Alloxan monohydrate, Vitamin E (VE), alpha lipoic acid (ALA) and reduced glutathione (GSH) (99.8%) were purchased from Sigma Chemical Co. USA. Other commercial kits used for the determination of glucose, cholesterol, triglycerides, total protein, creatinine, urea nitrogen, and uric acid were purchased from Stanbio Laboratory. USA. Urea and calcium kits were purchased from Quimica Clinica Aplicada S.A. Spain. Total lipids kit was purchased from Diamond diagnostics, Egypt. Tumor necrosis factor-α (TNFα) kit (purchased from CytImmune Sciences, USA). Total nitrate and nitrite kit (purchased from Assay Designs, USA) Insulin kit (purchased from LINCO Research, USA). Hepatic triglycerides, and total lipids measurements were performed in Biochemistry department, Faculty of medicine, Cairo University, Cairo, Egypt. Ellman's reagent, [5,5'-dithiobis-(2-nitrobenzoic acid)] (DTNB), was obtained from Aldrich Chemical Company. All reagents were of the highest purity commercially available.

Animals and Experimental Design

After one week of acclimation, male albino rats weighing 150-190 g were randomly divided into 4 groups (10 rats/group) as follows: Group A (Saline control) was normal and injected intraperitoneally (i.p.) with saline. Group B (Antioxidant-treated control) was injected i.p. daily by a mixture of VE (40 mg/kg wt) (Koya *et al*., 2003) and ALA (10 mg/kg wt) (Barbara *et al*., 2005). Group C (Diabetic) was injected i.p. by a single dose of alloxan (125 mg/kg wt) (Trivedi *et al*., 2004). Group D (Treated-diabetic) was injected with alloxan (125 mg/kg wt) and then injected i.p. daily by a mixture of VE (40 mg/kg wt) and ALA (10 mg/kg wt).

The powder of ALA was mixed with sterile saline in the dark bottle, and 5 N NaOH drop wise was added until the suspension dissolved (Barbara *et al*., 2005). The treatments were continued daily for 14 days and were started after 48 hrs of alloxan injection.

The diabetic state was assessed by measuring plasma glucose concentration after 48 hrs of alloxan injection. Rats exhibiting plasma glucose levels above 350 mg/dl were selected for the experiment and included in the study.

Blood and organs sampling

Blood samples of the fasted rats were collected from the medial retro-orbital venous plexus immediately with heparinized capillary tubes (Heparinized Micro Hematocrit Capillaries, Mucaps) under light ether anesthesia (Sanford, 1954). Then the blood was centrifuged at 3000 rpm for 15 min to separate plasma for different biochemical analyses. The animals were then decapi-tated under ether anaesthesia and tissue samples (liver, pancreas, and retina) were collected and preserved in -20°C for subsequent biochemical analyses.

Biochemical measurements

1.Determination of plasma metabolites

Plasma glucose levels were measured using the oxidase method described by Trinder (1959). Plasma lipid metabolites (cholesterol, triglycerides, total Lipids), protein metabolites (total Proteins, urea, BUN, creatinine), nucleic acids metabolites (uric acid), and some markers of oxidative stress (calcium ions, NO, TNFα) were assayed using commercial kits.

2.Determination of organs metabolites

Liver, pancreas and retinal homogenates were used for the estimation of reduced glutathione according to the method of Sedlak and Lindsay (1968). GSH in free supernatant was determined at 412 nm and expressed in mg g^{-1} tissue. Liver, pancreas and retinal lipid peroxide were

measured by a colorimetric reaction with thiobarbituric acid-positive reactant substances (TBARS) and was expressed in terms of the malondialdehyde (MDA) concentration by using 1,1,3,3-tetramethoxy propane as a standard at 535 nm according to the method described by Ohkawa *et al*. (1979). Catalase (CAT) activity in homogenate liver tissue was determined according to the methods of Beers and Sizer (1952) in which the disappearance of hydrogen peroxide is followed spectrophotometrically at 240 nm.

Statistical analyses

All data were expressed as mean ± standard error of the mean (S.E.). The statistical analyses were performed utilizing the software statistical package Microsoft Excel XP for data storage and producing histograms and SPSS version 13.0 for statistical evaluation. An independent t-test was used to examine the significance difference between groups. A statistical significance group difference (control and diabetic) was established at the 95% confidence level ($P < 0.05$).

Results

Plasma parameters

By the end of the two weeks period, Alloxan treatment induced a significant elevation ($P \leq 0.0001$) in plasma glucose and insulin levels of group C as compared to group A (Fig.1). The treatment of diabetic rats with antioxidant mixture achieved a significant reduction ($P \leq 0.0001$) by 73.6 % in the plasma glucose level and a significant increase ($P \leq 0.001$) by 44.7 % in plasma insulin level of group D as compared to group C. However, antioxidant mixture alone treatment was not shown to induce any significant changes on plasma levels of glucose and insulin when compared to group A values in spite of a 3.5 % decrease in glucose level and a 15 % increase in insulin level of group B ($P = 0.67$ and 0.06, respectively).

Induction of diabetes to male rats of group C by a single dose of alloxan produced a significant increase ($P \leq 0.003$) in plasma cholesterol, triglycerides and total lipids levels of rats. The results also revealed a significant increase ($P \leq 0.001$) in plasma urea, BUN, creatinine and uric acid levels. These changes were accompanied with significant decreases ($P \leq 0.002$) in plasma total proteins content when compared to group A (Table. 1). On the other hand with respect to treatment of alloxan diabetic rats with antioxidants mixture, the plasma metabolites levels of lipids, proteins and nucleic acids were reduced significantly except total proteins levels increased significantly ($P \leq 0.0001$) to be near to the normal levels of control group.

The early stage of diabetic induction by alloxan did not show any significant difference in plasma Ca^{+2} and NO levels of group C as compared to group A. Meanwhile, plasma TNFα of group C decreased significantly ($P \leq 0.0001$) as compared to group A. On the other hand, the antioxidant mixture treatment did not cause significant changes in plasma Ca^{+2} and NO levels in both group B and D as compared to group A. However, the treatment with antioxidant mixture attained a significant decrease ($P \leq 0.01$) in plasma TNFα level by 13.5 % in group B and by 6.5 % in group D as compared to group A (Table. 2).

Tissue parameters

Diabetic state in the early period (2 wks) of the present study produced a significant increase ($P \leq 0.01$) in hepatic triglycerides, total lipids (Fig. 2). Also, GSH content in both liver and retina elevated significantly ($P \leq 0.001$). Meanwhile, hepatic Ca^{+2} and pancreatic GSH content did not change significantly in alloxan diabetic rats of group C as compared to group A (Fig. 3). Conversely, antioxidant mixture treatment attained a significant decrease ($P \leq 0.03$) in GSH content of both liver and retina of group D while pancreatic GSH content increased significantly ($P \leq 0.03$) in group D as compared to group C. However, antioxidant mixture did not attain any significant changes in both pancreas and retina of group B but attained a significant decrease in liver GSH content of group B as compared to group A.

Alloxan induced a sustained decrease ($P \leq 0.02$) in the retinal and liver TBARS levels of group C as compared to group A.

Meanwhile, pancreatic TBARS levels of group C attained a significant increase ($P \leq 0.0001$) as compared to group A (Fig. 4). On the other hand, antioxidant mixture did not attain any effect on the retinal and liver TBARS levels of group D as compared to group C. Conversely, antioxidant mixture treatment attained a significant reduction ($P \leq 0.0001$) by 33.1 % on TBARS levels of group D as compared to group C. However, antioxidant mixture did not attain any significant changes in both liver and pancreas of group B but attained a significant decrease in retina TBARS levels of group B as compared to group A.

Liver catalase activity of group C decreased significantly ($P \leq 0.035$) as compared to group A after the induction of diabetes by alloxan alone (Fig. 5). However, antioxidant mixture treatment attained a significant increase ($P \leq 0.01$) in group D as compared to group C. Nevertheless, antioxidant mixture treatment did not attain any significant changes in group B as compared to group A.

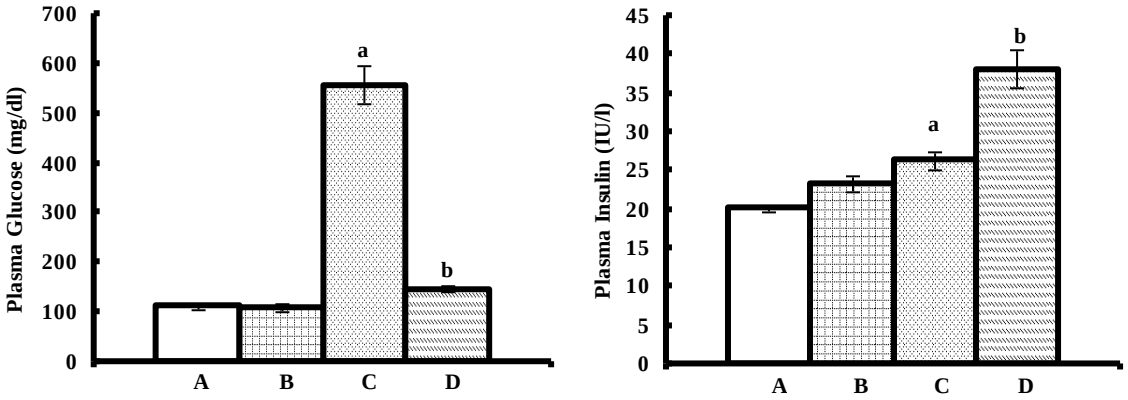

Fig. 1. Effect of antioxidant mixture of vitamin E (40 mg/kg wt) and ALA (10 mg/kg wt) on plasma glucose and insulin levels.

Values were represented as mean ± S.E. of 10 rats. Accepted level of significance is at $P \leq 0.05$. Saline control (group A), antioxidant control (group B), diabetic (group C) and treated-diabetic (group D). [a] values were statistically significant when group C was compared with group A and [b] values were statistically significant when group D was compared with group C.

Table (1): Effect of antioxidant mixture of vitamin E (40 mg/kg wt) and ALA (10 mg/kg wt) on plasma metabolic markers.

Plasma parameters	Groups			
	A	B	C	D
Cholesterol (mg/dl)	62.4 ± 4.8	61.5 ± 2.4	129.3 ± 6.98 [b]	70.6 ± 5.6 [c]
Triglycerides (mg/dl)	54.6 ± 3.4	55.2 ± 2.6	117.0 ± 9.1 [b]	77.1 ± 6.2 [c]
Total lipids (mg/dl)	217.3 ± 0.8	217.8 ± 0.7	441.2 ± 10.5 [b]	218.8 ± 0.6 [c]
Total proteins (g/dl)	5.6 ± 0.21	5.7 ± 0.34	4.5 ± 0.34 [b]	6.9 ± 0.3 [c]
Urea (mg/dl)	21.0 ± 1.4	20.9 ± 1.6	41.5 ± 3.02 [b]	25.5 ± 1.34 [c]
BUN (mg/dl)	17.27 ± 0.87	18.3 ± 1.0	28.9 ± 3.1 [b]	19.9 ± 0.8 [c]
Creatinine (mg/dl)	0.9 ± 0.05	0.7 ± 0.032 [a]	1.5 ± 0.07 [b]	0.7 ± 0.03 [c]
Uric acid (mg/dl)	3.2 ± 0.06	3.1 ± 0.03	3.8 ± 0.15 [b]	3.3 ± 0.04 [c]

Table(2): Effect of antioxidant mixture of vitamin E (40 mg/kg wt) and ALA (10 mg/kg wt) on plasma Ca^{+2}, No and TNF *α*.

Plasma parameters	Groups			
	A	B	C	D
Ca^{+2} (mg/dl)	7.3 ± 0.3	6.4 ± 0.27	7.3 ± 0.6	6.9 ± 0.3
NO (μmol/ml)	22.1 ± 0.7	24.9 ± 1.74	25.2 ± 2.6	30.1± 3.3
TNFα (ng/ml)	187.0 ± 4.4	161.7 ± 5.6 [a]	136.1± 1.9 [b]	127.3 ± 2.2 [c]

Values were represented as mean ± S.E. of 10 rats. Accepted level of significance is at $P \leq 0.05$. Saline control (group A), antioxidant control (group B), diabetic (group C) and treated-diabetic (group D). [a] values were statistically significant when group B was compared with group A, [b] values were statistically significant when group C was compared with group A, and [c] values were statistically significant when group D was compared with group C.

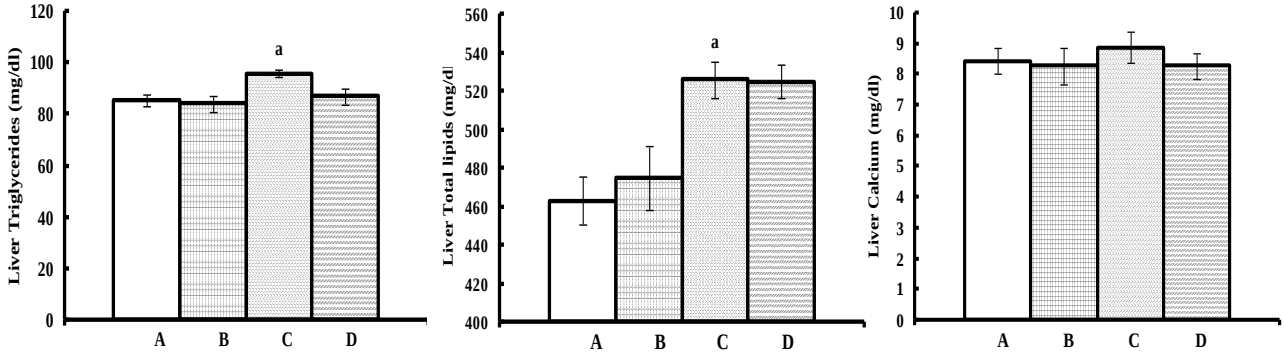

Fig. 2. Effect of antioxidant mixture of vitamin E (40 mg/kg wt) and ALA (10 mg/kg wt) on hepatic content of triglycerides, total lipids, and calcium.

Values were represented as mean ± S.E. of 10 rats. Accepted level of significance is at $P \leq 0.05$. Saline control (group A), antioxidant control (group B), diabetic (group C) and treated-diabetic (group D). [a] values were statistically significant when group C was compared with group A.

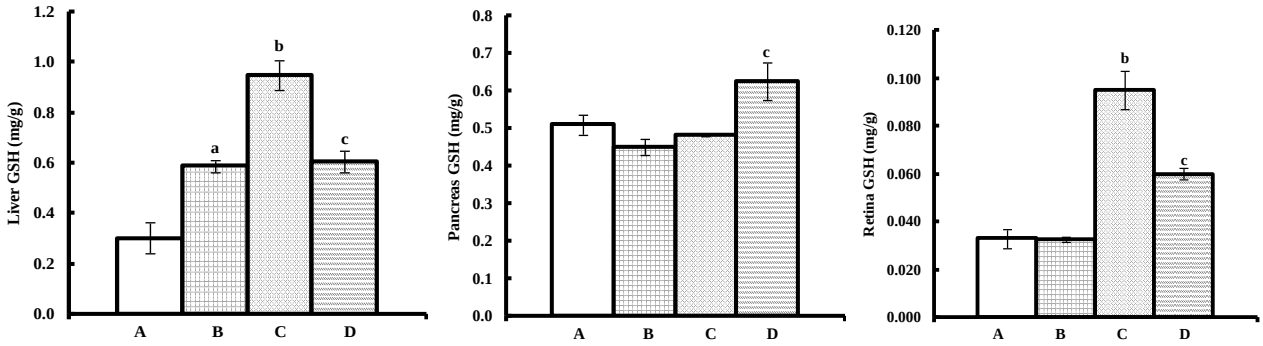

Fig. 3. Effect of antioxidant mixture of vitamin E (40 mg/kg wt) and ALA (10 mg/kg wt) on GSH content of liver, pancreas, and retina.

Values were represented as mean ± S.E. of 10 rats. Accepted level of significance is at $P \leq 0.05$. Saline control (group A), antioxidant control (group B), diabetic (group C) and treated-diabetic (group D). [a] values were statistically significant when group B was compared with group A, [b] values were statistically significant when group C was compared with group A, and [c] values were statistically significant when group D was compared with group C.

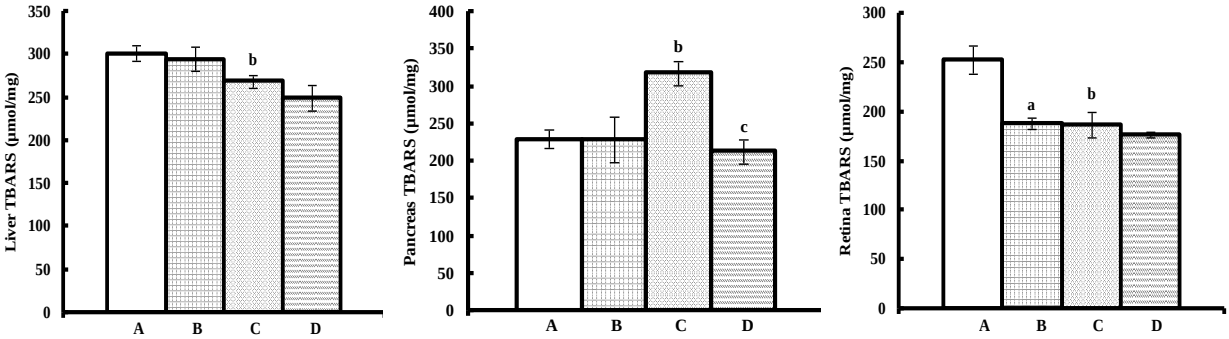

Fig. 4. Effect of antioxidant mixture of vitamin E (40 mg/kg wt) and ALA (10 mg/kg wt) on TBARS Level (µmol/mg) of liver, pancreas, and retina.

Values were represented as mean ± S.E. of 10 rats. Accepted level of significance is at $P \leq 0.05$. Saline control (group A), antioxidant control (group B), diabetic (group C) and treated-diabetic (group D). [a] values were statistically significant when group B was compared with group A, [b] values were statistically significant when group C was compared with group A, and [c] values were statistically significant when group D was compared with group C.

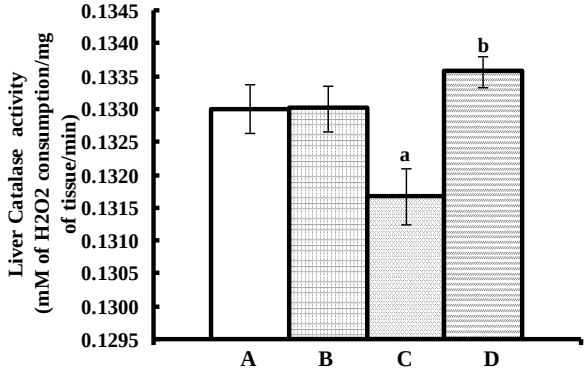

Fig. 5. Effect of antioxidant mixture of vitamin E (40 mg/kg wt) and ALA (10 mg/kg wt) on Liver CAT activity.

Values were represented as mean ± S.E. of 10 rats. Accepted level of significance is at $P \leq 0.05$. Saline control (group A), antioxidant control (group B), diabetic (group C) and treated-diabetic (group D). [a] values were statistically significant when group C was compared with group A and [b] values were statistically significant when group D was compared with group C.

Discussion

Generally, the animal models of diabetes exhibit characteristic features such as chronic hyperglycaemia, hyper-or normo-or hypoinsulinaemia (Srinivasan and Ramarao, 2007). An observation in this study correlates with the previous research finding (Momo *et al*., 2006 and Leite *et al*., 2007) in that the blood glucose levels increased significantly in alloxan–treated-diabetic rats.

The status of oxidative stress, cellular redox potential and their link to inflammatory cytokines appear to be the key elements mediating the cytotoxic effects of alloxan as well as the protective effects of the combined mixture of VE and ALA.

The etiology of impaired glucose metabolism in alloxan-treated rats seems to be multifactorial. It may reflect insulin resistance, since alloxan was reported to

induce insulin resistance or even abrogation of insulin action (Szkudelski *et al*., 1998 and Kandulaska *et al*., 1999). Moreover, it has a cytodestructive effects on pancreatic islets. The increase in plasma insulin level in alloxan-diabetic rats; compared to control level; can be attributed to the development of insulin resistance. Hyperinsulinemia is a marker of insulin resistance and has been demonstrated to be a strong predictor for type II diabetes (Henriksen *et al*., 1994). Therefore, alloxan could be a good candidate initiating effects that simulate both type I and type II diabetes. Insulin resistance may occur due to a defect in insulin binding caused by decreased receptor number or affinity, or defects at the level of effector molecules such as glucose transporters and enzymes involved in glucose metabolism (Kim *et al*., 2000).

Numerous studies have demonstrated a central role of oxidative stress in the etiology of diabetes and its complications (Evans *et al*., 2003). Meanwhile, the link between inflammatory cytokines and oxidative stress and their role in the pathogenesis of several diseases including diabetes is well established (Evans *et al*., 2003 and Byun *et al*., 2005). Altered tissue GSH and TBARS levels and the decrease in liver catalase activity clearly reflect oxidative stress in alloxan-diabetic rats. The apparent feature of diabetes is the impairment of energy metabolism, which is evident in alloxan-treated rats by the elevated levels of plasma creatinine, urea and BUN. These changes account for accelerated glycolysis and breakdown of creatinine phosphate which are the metabolic consequences of the activation of a back up system for the generation of ATP when the primary energy-forming pathway is impaired (Lehnenger, 1970). Actually, the study of Adamson and Billings (1992) indicated that the inflammatory cytokine TNFα caused a marked decrease in cellular ATP concentrations, which occurred secondary to effects on the glutathione pool. Thus, the altered levels of TNFα and GSH observed in alloxan-treated rats may contribute at least in part to impaired energy metabolism in these rats.

Glutathione in its reduced form (GSH) is the most powerful intracellular antioxidant (Packer *et al*., 2001 and Chaudhry *et al*., 2007), and the ratio of reduced to oxidized glutathione (GSH: GSSG) serves as a representative marker of the redox status of the cell (Exner *et al*., 2000). The marked increase in hepatic and retinal GSH in alloxan-treated rats observed after 14 days of alloxan treatment may indicate a compensatory defense mechanism against alloxan-induced oxidative stress. This compensatory mechanism was also evident by the increase in the plasma level of uric acid in the same rats. Uric acid is a potent antioxidant that scavenges reactive oxygen species (ROS) and reactive nitrogen species (RNS) (Ames *et al*., 1981 and Fang *et al*., 2002). The elevated GSH level seems to attain an initial reduction in its levels during the early phases of alloxan toxicity. A reduction in tissues GSH contents have been previously documented early after alloxan intoxication (Moustafa, 2004).

There are several lines of evidence to support the hypothesis that a strong link exists between TNFα and the GSH status which should impose a strong impact on glucose metabolism. It has been reported that persistent TNFα secretion could induce oxidative stress through modulation of GSH metabolism (Glosli *et al*., 2002). The current results would clearly support the existence of a close link between the GSH status and TNFα. The defense compensatory mechanism reflected by the elevated GSH levels (hepatic and retinal) in alloxan-treated rats was accompanied by reduced levels of their plasma TNFα emphasizing the inverse relationship between both elements. Considering the fact that TNFα is over produced in diabetes (DiPetrillo *et al*., 2003), and based on the solid inverse relation between GSH and TNFα. It may be conclude that TNFα was overproduced during early phases of alloxan treatment.

The current results showed that the hepatic and retinal levels of the oxidative stress marker; TBARS; goes parallel with the levels of plasma TNFα and at the same time had an inverse image to the tissue levels of GSH. This may support the hypothesized link between oxidative stress status and TNFα.

TNFα is known to cause cell injury by generation of oxidative stress (Morales *et*

al., 1997). Meanwhile, oxidative stress is documented to be a major contributor to insulin resistance in diabetes (Evans *et al*., 2003), aging (Campisi, 2001) and alloxan toxicity (Szkudelski, 2001). In mice, chronic exposure of cells or whole animals to TNFα induces insulin resistance, and treatment with soluble forms of TNFα receptors neutralizes this effect (Uysal *et al*., 1997). Moreover, resistin and TNFα have been shown to directly impair insulin signalling and consequently, insulin stimulated glucose uptake in muscle (Dyck *et al*., 2006). Thus, it can be concluded that augmented oxidative stress caused by alterations in GSH and TNFα status could initiate a broad spectrum of impaired cell signaling pathways including insulin signaling. Apparently, this effect may account for impaired glucose metabolism in alloxan-treated rats.

Since hyperglycemia was documented to be a main mechanism mediating the development of diabetes complications (Evans *et al*., 2003), normoglycemia was desired effect of any drug used either singly or in combination in the treatment of diabetes. Actually, the present data reveal that the combination of VE supplementation with ALA synergistically improved hyperglycemia in alloxan-treated rats, and ameliorated the impaired energy metabolism of the same rats. This was indicated by the corrected levels of creatinine, urea and BUN after antioxidant mixture treatment.

The mechanisms mediating the protective effects of VE and ALA are mainly based on their characteristic feature being antioxidants. Proposed mechanisms for this protection include:

1- Improvement of glucose metabolism

This comprises possible effects of VE and ALA on the following items which in fact are interrelated:

a. Oxidative stress and inflammatory cytokines

Because of the antioxidant properties of ALA, it is particularly suited to the prevention and/or treatment of diabetic complications that arise from an overproduction of reactive oxygen and nitrogen species. In addition to its antiox-idant properties, ALA increases glucose uptake through recruitment of the glucose transporters to plasma membranes, a mechanism that is shared with insulin-stimulated glucose uptake (Packer *et al*., 2001). Indeed, recent trials have demonstrated that ALA improves glucose disposal in patients with type II diabetes (Packer *et al*., 2001).

On the other hand, VE is the major lipid peroxidation chain-breaking antioxidant and is located in the lipid phase of the cell. When VE quenches a ROS, a VE radical is formed that must be recycled back to its reduced form to continue to provide antioxidant protection. Importantly, ALA is capable of recycling VE (Packer *et al*., 1997). In addition, ALA, reduced ALA (dihydrolipoic acid), and their metabolites also function as antioxidants. This is because of their capacity to quench ROS in both the aqueous and lipid phases of the cell (Packer *et al*., 1995), Moreover, ALA has also been reported to be an effective glutathione substitute, capable of increasing cellular glutathione levels and further improving the antioxidant status of the cell (Han *et al*., 1997). Actually, in adipocytes, ALA; being a thiol antioxidant; was reported to increase GSH content significantly (Moini *et al*., 2002). Pointing to the close link between GSH and TNFα, one suggested mechanism of ALA cytoprotective effects is to be its effect on TNFα which could be direct (Zhang & Frei, 2001 and Packer, 1998) or of course secondary to its GSH replenishing property. Meanwhile, VE improves the free radical defense system potential and prevents oxidative stress induced insulin resistance in rat L6 muscle cells (Vinayaga *et al*., 2006). Actually the current data show that VE and ALA treatment has corrected the levels of oxidative stress parameters GSH, TBARS and catalase in alloxan-diabetic rats. The decrease in tissue GSH in antioxidant-treated rats is an expected finding that supports the causal relationship between oxidative stress and the GSH status. That is, when oxidative stress was alleviated; due to antioxidant treatment; there was no need for the over synthesis of GSH in alloxan-diabetic rats.

b. Protein molecules and the protein manufacturing machinery

The major antidiabetic effects of VE and ALA may lie in their ability to affect

one or more of the effector molecules regulating glucose metabolism. Most of the components affecting glucose transport and metabolism are protein molecules. These include insulin receptors, glucose transporters, and enzymes among others protein molecules. Indeed, Guilliams (2002) reported that protein is the primary mediator of glucose transport into cells. Glucose transport is the initial step in glucose metabolism.

The membrane-associated insulin receptors are the initial elements mediating glucose transport, and the possible protection offered to them by the synergistic effects of both VE and ALA is of special importance. This is because glucose transport is the rate limiting step for overall glucose metabolism (Fink *et al*., 1986).

It is worthy to refer to the study of Moustafa *et al*. (1995) showing impaired glucose transport in adipocytes of aged hyperglycemic rats. Oxidative stress and enhanced lipid peroxidation in these cells were suggested to mediate this impairment. Therefore, it seem logical to suggest that alloxan-induced oxidative stress may result in alteration in the rate of glucose transport and/or one of the more distal intracellular processes affecting glucose metabolism.

Thus, it is recommended that any protective regimen against diabetes or its complications should be designed to act at this particular point. In fact, the two antioxidants used in the current study were carefully selected to serve this purpose. Both VE and ALA are lipid soluble, therefore, both can act to protect the cell membrane-associated receptors initiating the first and the most critical step in glucose transport. Being water soluble, ALA may have the feasibility to protect cellular components other than those associated with cellular membrane(s). Of particular importance is ALA expected ability to protect the DNA strand against free radical-induced damage. DNA fragmentation was proposed to be one of the mechanisms of alloxan toxicity (Sakurai and Ogiso, 1995). In addition, apart from being an important antioxidant, uric acid is the end product of nucleic acids breakdown (Scanlon and Sanders, 2007). Therefore, its elevated plasma levels in alloxan-diabetic rats may account for the accelerated DNA damage in these animals. In same time, its restored levels towards normal in antioxidant-treated animals may point to the protective capacity of LA on the DNA strand. Actually, the study of Garg *et al*. (2005) has shown that plasma uric acid levels were restored to near normal levels on VE supplementation in STZ-diabetic rats.

The synergistic action of ALA and VE would give them the capacity to protect other membrane(s)-linked components involved in protein synthesis e.g. ribosomes. Consequently, most of the compounds involved in glucose transport and metabolism could be protected via the synergistic and additive effects of VE and ALA. In addition, ALA may have a positive impact on protein metabolism secondary to its effect as an insulin-sensitizing agent. Actually ALA was found to increases insulin sensitivity in patients with type II diabetes (Jacob *et al*., 1999) and it is well known that insulin stimulates the incorporation of amino acids into protein (Mansour *et al*., 2002 and Guyton & Hall, 2006). Supporting to this hypothesis are the current data showing that combined treatment of VE and AlA has elevated the plasma protein levels significantly after being initially depleted in alloxan-diabetic rats. In addition, the restored levels of CAT activity in antioxidants-protected rats could be a result of the protection of VE and ALA on CAT molecule either directly or at its synthetic level through their protection of DNA molecule. An interesting observation is that both plasma proteins and liver CAT levels had exactly the same pattern in both alloxan-diabetic and antioxidant-protected rats. In addition, the noticeable rise in plasma insulin concentration in antioxidant-treated rats; compared to its level in diabetic rats; seems to account for the protective effects of VE and ALA on the protein synthesizing machinery of the pancreatic β-cells.

An additional mechanism proposed for the protein saving effects of ALA is maintaining the redox status of the cell. It has been reported that maintaining the redox potential of the cell is an essential factors for the prop er functioning of the protein manufacturing machinery of the cell (Hazelton and Calvin, 1980). Consequently, ALA being a GSH substitute may a play a

critical role in protecting the protein manufacturing machinery of the cell secondary to its effects in maintaining the redox status of the cell.

C. Lipid profile

It is important to emphasize the importance of the idea that lipid accumulation in skeletal muscle and liver leads to the development of insulin resistance. In experimental models, enhanced cholesterol levels and its subsequent deposition into liver mitochondria was found to induce selective decrease in the mitochondrial GSH stores. That was sufficient by itself to sensitize hepatocytes to TNFα mediated cell death (Fernandez-Checa, 2003). Its worthy to refer to the study of Yu *et al*. (2002) reporting that hyperlipidemia leads to increased uptake of fatty acids by muscle cells and production of fatty acid metabolites that stimulate inflammatory cascades and inhibit insulin signaling. Moreover, Jakus (2000) reported that elevated plasma levels of fatty acids suppress glucose uptake by interfering with the insulin receptor substrate proteins (IRS-1) signaling pathway. It follows that any perturbation that results in accumulation of intracellular fatty acids or their metabolites in muscle and liver may set off a cascade of events that leads to reduced IRS leading to insulin resistance in these tissues.

It seems logic to assume that the combination of the proposed initial TNFα overproduction, GSH depletion and lipid accumulation in the liver caused by alloxan treatment cooperate making cells more sensitive to alloxan poisoning. Therefore, the current data showed elevated levels of plasma and liver lipid parameters; which correlated with the well documented diabetes-associated hyperlipidemia (Bopanna *et al*., 1997 and Pari & Latha, 2002); may reflect a TNFα mediated mechanism contributing to insulin resistance and hyperglycemia in these rats.

Meanwhile, the corrected levels of lipid parameters in antioxidant–protected rats supports the hypothesis that this effect could be secondary to their primary role on TNFα and GSH status alleviating oxidative stress and sensitizing cells to insulin action. The anti-lipolytic effects of insulin are well documented (Guyton and Hall, 2006). Indeed, the effects of VE in ameliorating lipid profile in diabetic animals are well established (Karasu *et al*., 1997; Haidara *et al*., 2004 and Ozkan *et al*., 2005). In addition, ALA was found to activate AMP-activated protein kinase (AMPK) in skeletal muscle, resulting in enhanced fatty acid oxidation and reduced lipid accumulation (Lee *et al*., 2005). Evidence for the selective inhibitory effect of VE and ALA on TNFα comes from the current data showing that the corrected levels of oxidative stress parameters in antioxidant-protected rats were accompanied by a further remarkable reduction in plasma TNFα levels in the same rats.

2- Protection of insulin- secreting β-cells

The role of oxidative stress in mediating the cyto-destructive effects of alloxan on insulin-secreting β-cells is well established (Szkudelski, 2001), and the potential impact of inflammatory cytokines in this effect is of special importance (Rabinovich and Suarez-Pinzon, 1998). β-cells are highly vulnerable to free radical damage which has been attributed, at least in part, to the low activities of oxygen free radical scavenging enzymes in islet cells (Malaisse, 1982). In addition, alloxan was documented to exhibit a high affinity to the SH-containing cellular compounds including GSH (Szkudelski, 2001). These facts may explain the current data showing that pancreatic cells behaved differently in response to alloxan toxicity when compared to hepatic or retinal cells. The adaptive compensatory mechanism elevating cellular GSH in the liver and the retina above normal in alloxan-diabetic rats was not expressed markedly in the pancreatic cells of the same rats. Although GSH did not remain depleted after 14 days of alloxan treatment, yet it was not elevated (above normal) in pancreatic cells as it was in the liver and the retina. As expected, the level of pancreatic TBARS were not reduced in the pancreatic cells of alloxan-treated rats; actually elevated; contrary to what was seen in the liver and the retina.

The expected increase in plasma TNFα; at early stages of alloxan toxicity; may point to its anticipated negative impact on pancreatic β-cells. TNFα causes a selective inhibition of insulin release in rat

islets without causing cell death (Rabinovich *et al*., 1992). However, the highly vulnerable pancreatic cells to free radicals damage make them highly susceptible to ROS–mediated cyto-destructive effects of TNFα. Of course the GSH replenishing property of ALA; consequently its anti-TNFα effects; and the anti-oxidative capacity of its combination with VE may act to protect pancreatic β-cells against free radicals-induced damage. This was obvious by the reduced TBARS in the pancreatic β-cells of antioxidants-protected rats that was accompanied with elevated GSH levels in the same cells. Meanwhile, the remarkable increase in plasma insulin in alloxan-diabetic rats could be attributed at least in part the suppression of the selective inhibition of TNFα of insulin secretion.

Disturbances in calcium homeostasis have been reported to play key role in alloxan-induced ß cell damage (Park *et al*., 1995 and Kim *et al*., 2000). It has been proposed that, in liver cell necrosis, ion pump failure associated with depolarization of the membrane may result in the increase of an influx of calcium into the cell (Farber, 1982). A similar mechanism was reported to operate in alloxan–induced β-cells cytotoxicity (Szkudelski, 2001). Based on the development of oxidative stress in alloxan diabetic rats, one basic mechanism explaining the decreased catalase activity observed in these rats is the expected increase in cellular H_2O_2. The proposed increase in H_2O_2 could be a causative factor inducing β-cells damage in alloxan toxicity as suggested by Park *et al*. (1995). This effect was reported to be mediated through interfering with Ca^{+2} homeostasis (Park *et al*., 1995). Interestingly, is the findings that NO inhibits H_2O_2 degradation (Rauen *et al*., 2007) which could be of special significance in interpreting the present findings. It is noticed that plasma NO level was not decreased in alloxan diabetic rats as predicted from previous findings (Mohan and Das, 1998) showing decreased NO in alloxan-treated rats. Therefore, this relatively increased NO level at this time point of alloxan toxicity (14 days) could be one factor justifying the decreased catalase activity in alloxan-diabetic rats.

Consequ-ently, this may support the possible role of H_2O_2 in inducing β-cell damage in these rats. Meanwhile, increased Ca^{+2} was repor-ted enhance NO synthesis which means that the three elements, NO, H_2O_2 and Ca^{+2} have co-operativity in their cytotoxic action. The non significant increase in tissue Ca^{+2} shown in alloxan treated animals of the present study doesn't exclude the possibility of its initial detectable rise in earlier phases of alloxan toxicity. Actually, other studies have shown an increase in NO production in early diabetes e.g. Pieper (1998), thus proposed to be one factor of elevating tissue Ca^{+2} at this stage.

The unaffected levels of NO and Ca^{+2} in antioxidant–treated rats shown in the present study could mean that the synerg-istic action of these two antioxidants on these two particular parameters didn't attain its full impact at this particular point of treatment. In fact, it has been found that supplementation of VE (Vasdev *et al*., 2000) and ALA (Vasdev *et al*., 2002) had a positive role in lowering elevated cytosolic Ca^{+2}.

Summing up, the current findings favor the concept that oxidative stress may be a final common pathway in the develop-ment of diabetes. Deteriorative changes have been provoked in early diabetic stage (2 weeks) in the metabolism of carbohy-drates, lipids, proteins, and nucleic acids. In addition, the study revealed that alloxan-induced destructive changes were more obvious in the pancreas than in the retina and the liver. This was evidenced by the visible deterioration in antioxidant status and the marked increase in oxidative stress markers in the pancreas, compared to liver and retina. Furthermore, it is clear that the strategy of combining different antioxid-ants, which protect each other and act together at different levels of the protein molecules and lipid profile, would alleviate inflammatory cytokines and oxidative stress status.

Therefore, this strategy may provide a therapeutic tool in cases of oxidative stress-induced cellular damage including diabetes. In general, prophylaxis with a VE-ALA mixture may be valuable in reducing the risks of diabetes and its complications.

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الدور الوقائي المحتمل لكل من فيتامين ه وحمض ألفا ليبويك فى مواجهة التغيرات المبكره في الجرذان البيضاء المصابة بالسكري لحقنها بالألوكسان

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أجريت الدراسة الحالية لدراسة تأثير مرض السكري على الأيض السكريات والدهون والبروتينات والأحماض النووية، بالإضافة إلى معرفة مدى إمكانية تأثيره على فعالية مضادات الأكسدة و مدى قدرته على إحداث حالة الإجهاد التأكسدي في الجرذان المعاملة بمركب الألوكسان (125ملجم/كجم في التجويف البروتوني) وكذلك استكشاف الدور المحتمل للمعالجة بخليط من مضادات التأكسد المكون من فيتامين هـ (40 ملجم/كجم/يوم) و حمض ألفا ليبويك (10 ملجم/كجم/يوم) بعد حقنها في التجويف البروتوني لمدة 14 يوم على التوالي.

وقد أوضحت نتائج تلك الدراسة أن هناك زيادة دالة إحصائيا في محتوى المصل من السكر والإنسولين والأحماض الدهنية (الدهون الثلاثية، الكوليستيرول، الدهون الكلية) والمواد النيتروجينية الناتجة من تكسير البروتينات (البولينا والكرياتينين) والمواد الناتجة من تكسير الأحماض النووية (حمض البوليك)، محتوى الكبد وشبكية العين من الجلوتاثيون ومحتوى الكبد من الدهون الثلاثية، الدهون الكلية في الجرذان المصابة بالسكري. هذه التغيرات كانت مصحوبة بالنقص المعنوي في محتوى المصل من البروتينات، العامل تي. إن ألفا نيكروسيس (TNFα) ومحتوى الكبد من إنزيم الكتاليز (CAT)، بينما لم يحدث تغير معنوي في محتوى المصل من كل من أيونات الكالسيوم وأكسيد النيتريد (NO) و محتوى البنكرياس من الجلوتاثيون (GSH) ومحتوى الكبد من أيونات الكالسيوم في الجرذان المصابة بالسكري مقارنة بالمجموعة الضابطة. أما بالنسبة لدلائل الإجهاد التأكسدي (TBARS)؛ فقد حدث زيادة معنوية في محتوى البنكرياس من هذه المواد، ولكن من الجهة الأخرى حدث نقص معنوي في نسبة TBARS فى كل من الكبد وشبكية العين في الجرذان المصـــابة بالسكري مقارنة بالمجموعة الضابطة.

أما بعد المعالجة بخليط من مضادات التأكسد؛ فقد لوحظ أنها أدت إلى انخفاض نسب السكر، الدهون الثلاثية، الكوليستيرول، الدهون الكلية، البولينا، الكرياتينين، حمض البوليك والعامل تي إن ألفا نيكروسيس في مصل الجرذان المصابة بالسكري ومحتوى الكبد وشبكية العين من الجلوتاثيون بالإضافة إلى نسبة دلائل الإجهاد التأكسدي في البنكرياس لتكون قريبة من النسب الطبيعية الموجودة بالمجموعة الضابطة، كما لوحظ زيادة معنوية فى الإنسولين، البروتين بمصل الجرذان المصابة بالسكري، محتوى الكبد من إنزيم الكتاليز ومحتوى البنكرباس من الجلوتاثيون في الجرذان المصابة بالسكري. علاوة على ذلك، لم تستطع المعالجة بالخليط المستخدم في التأثير على نسبة أيونات الكالسيوم وأكسيد النيتريد بمصل الجرذان المصابة بالسكري وأيضاً لم تؤثر على محتوى الكبد من الدهون الثلاثية، الدهون الكلية وأيونات الكالسيوم بالإضافة الى نسبة دلائل الإجهاد التأكسدي في كل من الكبد والشبكية في الجرذان المصابة بالسكري.

من كل ما سبق تكون النتائج قد أكدت على أن مرض السكري خلال فترة الدراسة (14 يوم) قد أدى إلى حدوث حالة الإجهاد التأكسدي والأثار الواضحة على البنكرياس مقارنة بكل من الكبد وشبكية العين في الجرذان المصابة بالسكري والتى قد أدت بدورها إلى حدوث تغيير معنوى فى مستوى الأيض للسكريات والدهون والبروتينات والأحماض النووية. من الجهة الأخرى، كما تبين من الدراسة الحالية التأثير الفعال للخليط العلاجي كمواد مضادة للأكسدة في منع التعقيدات المصاحبة لمرض السكري بواسطة تقليل الإجهاد التأكسدي وتحسين مستوى الأيض للسكريات والدهون والبروتينات والأحماض النووية وزيادة القدرة الفعالة لمضادات الأكسدة في المصل، الكبد، البنكرياس وشبكية العين لتصل إلى معدلات أقرب من النسب الطبيعية الموجودة بالمجموعة الضابطة.