EVALUATION OF ANTIOXIDANT STATUS IN STREPTOZOTOCIN-INDUCED DIABETIC RATS BY FERRIC REDUCING ABILITY OF PLASMA ASSAY

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Abstract - Enhanced oxidative stress in diabetes mellitus may contribute to the pathogenesis of diabetic complications. In this article, the stress oxidative generation has been studied in experimental diabetes by ferric reducing ability of plasma assay, a sensitive and simple method, and by other oxidative damage markers. The ferric reducing ability of plasma values as a total antioxidant capacity were significantly decreased at the 3th and 4th weeks of study ($p<0.02$), while the thiobarbituric acid reacting substances (TBARS) levels in plasma were increased at the 3rd and the 4th weeks ($p<0.05$). The plasma carbonyl groups (PCG) were not affected, and total thiol groups (TG) were significantly decreased at the 4th week ($p<0.02$).

In conclusion the present study suggests that hyperglycemia in diabetes leads to oxidative stress, as shown by ferric reducing ability of plasma assay. This method is rapid, simple and economic.

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Key Words: Oxidative stress, antioxidants, FRAP, TBARS, PCG, TG, streptozotocin, diabetes mellitus

INTRODUCTION

The generation of free radicals and reactive oxygen species (ROS) has been reported to be increased in diabetic patients and experimental models (1,3), and this appears to be involved in the pathogenesis of diabetic complications (4,5).

Normally, ROS production is an integral part of aerobic metabolism in biological systems, but these potentially harmful molecules are inactivated or scavenged by a large group of antioxidants (6). The imbalance between ROS production and antioxidant status is often referred to as oxidative stress (7). There are growing evidences showing that this situation may be involved in the pathogenesis of more than 100 disease states (8).

Hence, great efforts have been made to evaluate oxidative stress and antioxidant defense systems. Generally, evaluation of oxidative stress is done by determining damaged biological products or assessing changes in antioxidants level (Vit. E, C and β-carotenes) (9).

Due to the difficulty in measuring each antioxidant component separately or because of the interaction between different antioxidants (10), several methods have been developed to measure the total antioxidant power of the biologic samples (11,14). The most employed procedures in this respect are trolox equivalent antioxidant capacity (TEAC) assay of Miller et al (commercially available kits) and the total radical trapping antioxidant potential (TRAP) assay of Loeke et al (14).

Recently, the ferric reducing ability of plasma (FRAP) assay has been developed by Benzie and Strain (13), which is more economical; its reagents are easy to prepare, and its procedure is rapid. The method could also be applied in clinical laboratory.

The purpose of this study was to assess oxidative stress generated in streptozotocin induced diabetic rats through employing FRAP assay. Moreover, in order to support our results, we analyzed the degree of lipid peroxidation and protein oxidation in plasma as general markers of oxidative stress.

MATERIALS AND METHODS

Tripyridyl-s-triazine (TPTZ), thiobarbituric acid (TBA) and Dinitrophenylhydrazine (DNPH) were purchased from Merk (Darmstadt, Germany). 2,2 dithiobisnitrobenzoic acid (DTNB) and di-t.butylhydroxytoluene (BHT) were obtained from Sigma Chemical Co. (St. Louis-USA). 1,1,3,3 - tetramethoxypropane was provided by Reidel-de Haen Co. (Germany) and Streptozocin (STZ) was obtained from Upjohn Co. (USA). All other reagents used were of analytical grade.

Animals: Male Sprague-Dawley rats (160-200 g) were purchased from Razi institute (Teh. Iran). Diabetes was induced by a single intraperitoneal (IP) injection of STZ (50 mg/kg body weight) which was

prepared in citrate buffer (pH = 4.0). The control rats were injected with buffer alone. Control and diabetic rats were weighted every two days and diabetes was confirmed three days after STZ administration by determination of plasma glucose (glucose oxidase method) using samples obtained from tail vein. Final samples for complete analysis were collected in tubes containing EDTA by cardiac puncture at weekly intervals for 4 weeks.

Oxidative stress parameters: We used FRAP assay for total antioxidant capacity of plasma. The method is based on the reduction of a Fe^{+++}-TPTZ complex to Fe^{++}-TPTZ complex form, which has an absorbance at 593 nm and color formation linearly related to the amount of reductants (13). The method of Satoh K. as previously described was used for measuring lipid peroxide (15) using 1,1,3,3, tetramethoxypropane as a standard. BHT was added to the medium to prevent the amplification of peroxidation. To evaluate protein oxidation, plasma carbonyl group content was measured by reaction with DNPH and it was calculated using the molar absorption coefficient of 22000 M^{-1} cm^{-1}. The values are expressed in nanomoles of carbonyl groups per mg protein. The amount of proteins were quantitated by reading the absorption at 280 nm and calculated from a bovine serum albumin (BSA) standard curve (0.25-2.0 mg/ml) (16). A spectrophotometric assay based on DTNB (Ellman's reagents) was used to measure protein thiol groups and glutathione in plasma (17).

The results are expressed as Mean ± SEM. Student's t-test (unpaired data analysis) and Pearson's coefficient test were used to determine significance of difference and correlation between the two groups, respectively. P values less than 0.05 were considered to be significant.

RESULTS

Plasma glucose and body weight for the experimental groups are summarized in table 1. The results showed that plasma glucose levels were significantly increased (3.4 folds) in the diabetic group starting from 1st week as compared to the control group ($p<0.001$). On the other hand, there was progressive increase in body weight of control rats (39.1% after 4 weeks), but no difference was seen in diabetic rats. However, there was a significant difference between diabetic and control groups at 1st, 2nd, 3rd and 4th weeks ($p<0.005$). The time-course values for FRAP assay are shown in Fig. 1. Diabetes induced by STZ caused considerable decrease in this parameter. These changes were more evident at 3rd week (346.0 ± 13.1, vs 391.0 ± 8.19, n = 5 $p<0.05$) and 4th week (316.6 ± 17.9 vs 402.0 ± 17.6, n = 5; $p<0.005$).

The term thiobarbituric acid reacting substances (TBARS) is applied for lipid peroxides. The plasma TBARS levels are illustrated in Fig. 2. The results indicated that TBARS levels were increased in diabetic group as compared to control group, which were significant at 3rd week (3.83 ± 0.22 vs 3.09 ± 0.15, n=5; $p<0.02$) and 4th week (3.90 ± 0.22, vs 3.10 ± 0.19, n=5 $p< 0.02$).

Plasma carbonyl groups as a marker of protein oxidation were not significantly affected as shown in Fig. 3. The levels of thiol groups showed significant reduction in diabetic group at 4th week (272.0 ± 12.9, vs 343.2 ± 17.7, n = 5, $p<0.01$) compared to control group (Fig. 4).

There was a negative correlation between FRAP values and TBARS levels ($r = 0.77$, $p<0.008$), while a positive correlation was present between FRAP values and thiol groups ($r = 0.70$, $p< 0.02$) after 4 weeks.

Table 1. Changes in plasma glucose levels and body weight in control and STZ - induced diabetic rats.

Duration (week)	Plasma glucose levels (mg/dl)		Body weight (g)	
	Control	Diabetic	Control	Diabetic
0	107.2 ± 5.6	107.2 ± 5.6	182.8 ± 6.3	182.8 ± 6.3
1	103.4 ± 5.6	309.2 ± 28.5*	214.2 ± 8.5	185.6 ± 6.3*
2	107.8 ± 5.0	351.4 ± 23.8*	240.8 ± 4.2	192.4 ± 6.7*
3	102.4 ± 5.5	316.4 ± 31.5*	274.8 ± 4.1	186.6 ± 6.6*
4	99.4 ± 4.7	358.0 ± 23.9*	300.2 ± 7.6	183.6 ± 7.0*

Each value represents the mean ± SEM (n = 5)

* $P < 0.05$ versus control

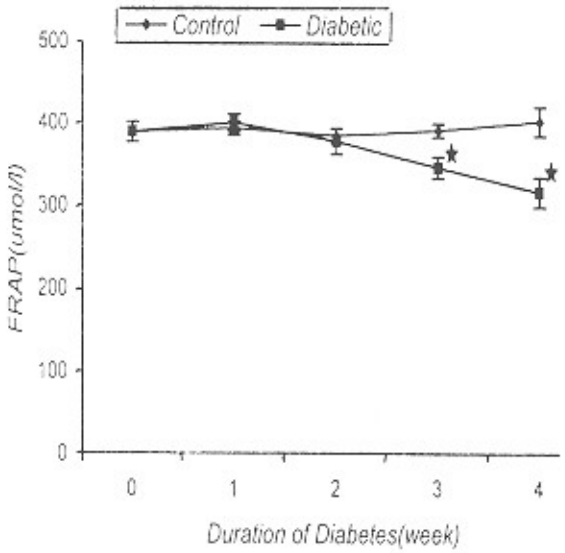

Fig. 1. Changes in Ferric Reducing Ability of Plasma (FRAP) in control and STZ-induced diabetic rats. The results are expressed as Mean ± SEM (n=5).
* p< 0.05 versus control

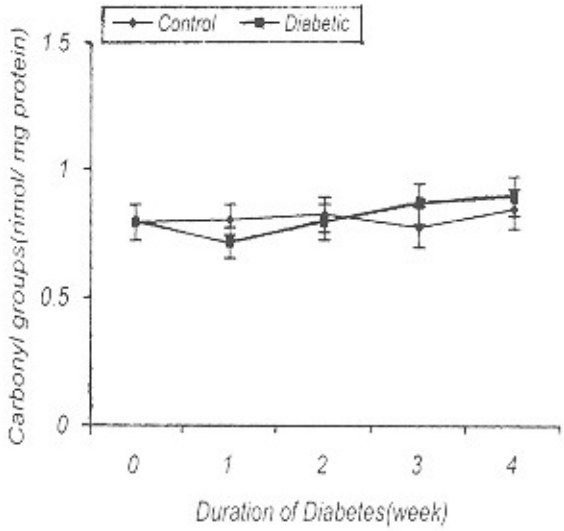

Fig. 3. Changes in Plasma carbonyl groups in control and STZ-induced diabetic rats. The results are expressed as Mean ± SEM (n=5).
* p< 0.05 versus control

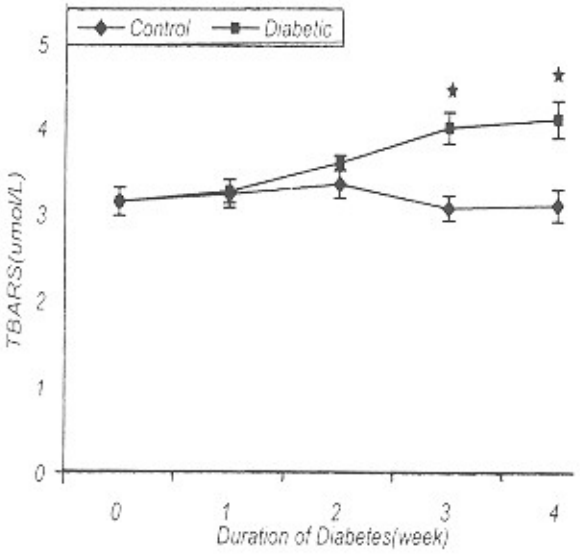

Fig. 2. Changes in thiobarbituric acid substances (TBARS) levels of plasma in control and STZ-induced diabetic rats. The results are expressed as Mean ± SEM (n=5).
* p< 0.05 versus control

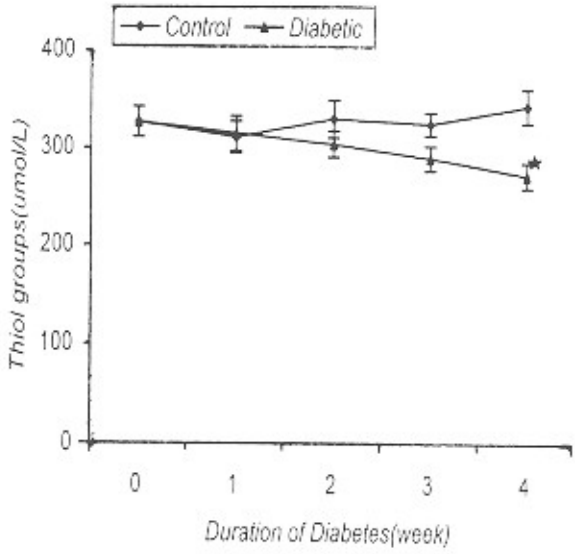

Fig. 4. Changes in thiol groups of plasma in control and STZ-induced diabetic rats. The results are expressed as Mean ± SEM (n=5).
* p< 0.05 versus control

DISCUSSION

The data reported in this study showed the presence of an oxidative stress in STZ-induced diabetic rats, which was in accordance with those of previous reports (18,20). To evaluate oxidative stress, we used FRAP method which provided a sensitive and simple assay for total antioxidant activity of biological samples such as plasma.

To our knowledge, there are no previously published data of FRAP values from STZ-induced diabetic rats. This method measures the reducing power of the most important antioxidants such as vitamin C, vitamin E, uric acid and bilirubin. However the method is not applicable to evaluation of the reducing power of glutathione and thiol groups (13). The decrease in FRAP values at the 3rd week (11.5%) and at the 4th week (21%) in diabetic rats may be due to consumption of antioxidants during the free radial scavenging process as diabetes is associated with increased oxidative stress.

Lipid peroxidation is another marker of oxidative stress in which plasma TBARS shows elevated levels at the 3rd and 4th weeks of diabetes. Our data are in agreement with numerous reports of an increase in plasma peroxidation products in diabetes mellitus (21,22). Excessive lipid peroxidation in the plasma could be due to uncontrolled production of reactive oxygen species. Decreased efficiency of antioxidant defence systems which contribute to free radical scavenging could be considered as another mechanism of lipid peroxide generation (23). The inverse correlation between FRAP values and TBARS levels confirm this situation. TBARS evaluation is not a very specific method, however it is the most frequently opted one in literature (9). A sensitive indicator of oxidative damage to plasma proteins is the decline of protein thiol groups. Our results show that there is a significant decrease in level of plasma thiol groups at the 4th week after administration of STZ. Most of the plasma thiol groups are protein associated. In addition, plasma contains small amount of glutathione. Decrease in the thiol group levels may be due to their consumption in scavenging free radicals or decrease in protein levels, specially albumin (24).

Other marker of protein oxidation is carbonyl group content. There is no significant difference for plasma carbonyl groups in the diabetic rats in comparison with the control rats. This may be due, to inadequate diabetic duration. Another assumption which can be made is the dependence of carbonyl group production on transition metals, but in plasma there are many protein and other molecules to bind with the free transition metals (25).

Several mechanisms have been proposed by which hyperglycemia could increase the generation of free radicals or impair their scavenging in biological systems. The main mechanisms are autoxidation of glucose, glycosylation of antioxidant enzymes and other proteins, intracellular activation of polyol pathway, alteration of glutatione redox system and increased production of free radicals at the mitochondrial levels (4,5).

Taken together, oxidative stress generation in STZ-induced diabetic rats is shown by FRAP assay and is confirmed by TBARS and thiol group measurement. The FRAP assay is inexpensive and carried out easily, in contrast to other methods which require highly valuable and sophisticated equipment.

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