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RESEARCH ARTICLE

Oral Zinc Supplementation Protects Rat Kidney Tissue from Oxidative Stress in Diabetic Rats [1]

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Summary

Zinc (Zn) is a trace element possessing a wide range of functions and antioxidant properties. This study was undertaken in order to illuminate the conflicting data on the status of zinc in diabetes, present in literature. Female Swiss albino rats were randomly divided into 4 groups: Group I, control; Group II, control + zinc sulfate; Group III, streptozotocin (STZ)-diabetic; Group IV, STZ-diabetic + zinc sulfate. Diabetes was induced by intraperitoneal injection of STZ (65 mg/kg body weight). Zinc sulfate was given daily by gavage at a dose of 100 mg/kg body weight every day for 60 days to Groups II and IV. At the last day of the experiment, rats were killed under anesthesia, kidney tissue was taken and homogenized. Antioxidant enzyme activities such as catalase (CAT), glutathione reductase (GR), glutathione peroxidase (GPx), glutathione-S-transferase (GST), superoxide dismutase (SOD) and myeloperoxidase (MPO), were determined in tissue homogenates as well as protein carbonyl content (PCC). Carbonic anhydrase (CA) was also determined as a functional enzyme for kidney. It was shown that kidney tissue antioxidant enzyme activities which were significantly impaired in the untreated diabetic group, were reversed in zinc treated diabetic groups, thus showing the beneficial effect of Zn treatment in diabetes via its antioxidant effect.

Keywords: *Zinc, Oxidative stress, Kidney, Diabetes mellitus, Antioxidant enzymes*

Ağızdan Çinko Verilmesi Diyabetik Sıçanlarda Böbrek Dokusunu Oksidatif Hasara Karşı Korur

Özet

Çinko (Zn), çok sayıdaki fonksiyonlarının yanı sıra antioksidan özellik de gösteren bir eser elementtir. Bu çalışma, bilimsel kaynaklarda, çinkonun diyabetteki etkisi ile ilgili çelişkili verileri aydınlatmak amacıyla gerçekleştirilmiştir. Swiss albino ırkı dişi sıçanlar 4 gruba ayrılmıştır: Grup I, kontrol; Grup II, kontrol + çinko sülfat, Grup III, streptozotosin (STZ)-diyabetik; Grup IV, STZ-diyabetik + çinko sülfat. Diyabet, intraperitoneal STZ (65 mg/kg) injeksiyonu ile oluşturulmuştur. Çinko sülfat, 60 gün süreyle, her gün 100 mg/kg oranında, gavaj yoluyla Grup II ve IV'e verilmiştir. Deneyin son gününde, sıçanlar anestezi altında kesildikten sonra böbrek dokuları alınarak homojenize edilmiştir. Doku homojenatlarında katalaz (CAT), glutatyon redüktaz (GR), glutatyon peroksidaz (GPx), glutatyon -S-transferaz (GST), superoksit dismütaz (SOD) ve miyeloperoksidaz (MPO) gibi antioksidan enzimlerin aktiviteleri ve protein karbonil miktarı (PCC) saptanmıştır. Ayrıca, böbrek için fonksiyonel bir enzim olan karbonik anhidraz (CA) aktivitesi de tayin edilmiştir. Diyabetik grupta anlamlı olarak değişiklik gösteren antioksidan enzim aktivitelerinin, çinko sülfat ile muamele edilmiş sıçanlarda iyileşme gösterdiği saptanmıştır. Sonuç olarak çinkonun diyabetteki olumlu etkisinin antioksidan etkisi nedeniyle olabileceği ileri sürülmüştür.

Anahtar sözcükler: *Çinko, Oksidatif stres, Böbrek, Diabetes mellitus, Antioksidan enzimler*

INTRODUCTION

Diabetes is a major cause of vascular complications affecting heart, kidney, retina and peripheral nerves. Hyperglycemia leads to oxidative stress that plays an important role in vascular degenerative lesions observed in diabetes [1].

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Streptozotocin (STZ) is a naturally produced antibiotic from *Streptomyces achromogenes* which has been widely used to induce diabetes in experimental animals [2]. It causes the selective destruction of pancreatic β-cells, probably by a free-radical-mediated mechanism.

Zinc (Zn) is a microelement required for the activity of nearly two hundred enzymes and is considered essential for cell division, DNA and protein biosynthesis [3]. The relation of plasma and urine Zn levels to diabetes mellitus has been investigated in several studies. Although hyperzincuria appears to be a common finding in most diabetic subjects [4], there are controversial findings about plasma Zn concentrations in diabetes [5]. Zn status is decreased in blood and tissues in most type 2 diabetic patients [1,3]. Zn levels in plasma, lymphocytes, granulocytes and platelets have been found lower in diabetic subjects in comparison to controls and it was concluded that diabetic patients are Zn deficient [3]. Zn metabolism seems to be altered in diabetic patients as well as in diabetic animals, and Zn supplementation has been shown to exhibit beneficial effects in diabetic animals and humans, which indicates that this metal might qualify as a future therapeutic intervention in diabetes mellitus [6-11]. This hypothesis is supported by the identification of zinc transporter (ZnT)-8, a protein responsible for zinc regulation, and by the proposed involvement of metallothionein (MT) another protein contributing to zinc homeostasis, in diabetes and its complications. In rats made diabetic by administration of STZ, a model of Type 1 diabetes mellitus, the zinc concentration in liver and kidney was increased, possibly due to an increase in MT, which could be observed in diabetic animals or the elevated zinc levels in these animal models could be explained by the sudden destruction of pancreatic β-cells by STZ, thus causing the release of zinc that was stored in β-cells [11].

Zn has numerous targets to modulate insulin activity, including its antioxidant capacity. Shisheva et al.[12], have concluded that Zn^{2+} mimics several actions of insulin both *in vitro* and *in vivo* by a mechanism unrelated to insulin. The insulinomimetic effect of Zn complexes have been studied in last years and the pharmacology of new complexes reported [13].

Superoxide dismutase (SOD) is the first antioxidant enzyme to deal with oxidative free radicals by accelerating the dismutation of superoxide to hydrogen peroxide, while catalase (CAT) is a peroxisomal heme protein that catalyses the removal of hydrogen peroxide formed during the reaction catalyzed by SOD. Thus, SOD and CAT act as mutually supportive antioxidative enzymes, which provide protective defense against reactive oxygen species (ROS). Any increase in SOD activity is beneficial in the event of increased free radical generation. However, a rise in SOD activity, without a concomitant rise in the activity of CAT and/or glutathione peroxidase (GPx) might be detrimental since SOD generates hydrogen peroxide as a metabolite, which must be scavenged by CAT or GPx.

Glutathione antioxidant system plays a fundamental role in cellular defense against reactive free radicals and other oxidant species. It consisted of reduced glutathione and an array of functionally-related enzymes, of which glutathione reductase (GR) is responsible for the regeneration of reduced glutathione (GSH), whereas GPx and glutathione-S-transferase (GST) work together with GSH in the decomposition of hydrogen peroxide or other organic hydroperoxides [14,15].

Kupffer cell myeloperoxidase (MPO) may be an important source of oxidative damage during tissue injury [16]. We have also measured tissue MPO activity to evaluate the degree of tissue infiltration by neutrophils.

Oxidative damage to several of amino acid residues and/or to the backbone of proteins can generate carbonyl products. Indeed, measurement of protein carbonyls has been used as a sensitive assay for oxidative damage to proteins, partly because it measures several different consequences of oxidative damage [17].

Carbonic anhydrase (CA), is a cytosolic enzyme present in most tissues of higher vertabrates and catalyzes the CO_2/HCO_3^- interconversion. It is speculated that changes in the activity of CA, may be of fundamental importance in the regulation of intracellular pH for the basic control of metabolism in diabetes mellitus. The altered CA activity could change an intracellular ion imbalance that might cause insulin resistance, which in turn might lead to type II diabetes [18].

Though the mechanism laying beyond the beneficial effect of Zn in diabetes has been explained through its insulinomimetic effect [13], the beneficial effect of Zn supplementation in non-diabetic animals *via* amelioration of the antioxidant balance has also been reported [19,20]. In addition, it is known that in diabetes, impairement of antioxidant defense system affects kidney tissue, leading to nephropathy. Nevertheless, the pathophysiology of diabetic nephropathy is not well defined. It is reported that high glucose directly increases hydrogen peroxide production by mesangial cells and lipid peroxidation of glomerular mesangial cells [21]. Regarding these hypotheses, in order to elucidate the mechanism of action of Zn, the present study intends to evaluate the effect of Zn treatment on the antioxidant parameters in the kidney tissue of diabetic rats.

MATERIAL and METHODS

Animals and Tissue Preparation

The experiments were approved and supervised by Animal Care and Use Comittee of Istanbul University. Female Swiss albino rats weighing 150-200 g were

used throughout the study. The animals were 6-6.5 months old and clinically healthy. The rats were fed with standard laboratory pellet chow with water *ad libitum*. The experimental period was maintained at constant laboratory temperature and the rats were submitted to 12 hours light/dark cycles.

The animals were divided into 4 groups:

Group I. Control (intact) animals (n=5).

Group II. Control animals given zinc sulfate (n=6).

Group III. STZ-induced diabetic animals (n=6). Diabetes was induced by intraperitoneal injection of STZ in a single dose of 65 mg/kg body weight. Freshly prepared solution was made by dissolving STZ in cold 0.01 M sodium citrate-HCl buffer.

Group IV. STZ-induced diabetic animals given zinc sulfate (n=9).

Zinc sulfate heptahydrate (Merck) was given to groups II and IV by gavage at a dose of 100 mg/kg body weight, every day, for 60 days. At the last day of the experiment, rats were killed, kidney tissues were taken, homogenized using Art-MICCRA D-1 homogenator by means of a glass homogenizer in cold saline (14.500 rpm) to make a 10% (w/v) homogenate. The homogenates were centrifuged at 13.000 rpm for 5 min (Megafuge Hereaus1.0R) and the clear supernatants were used for enzyme and protein analysis.

Biochemical Assays

Blood glucose levels after 18 h fasting were estimated by the o-toluidine method as described previously [10]. Each animal with a fasting blood glucose concentration above 200 mg/dL was considered diabetic.

All the assays were performed using the kidney tissue homogenate in appropriate dilutions. Shimadzu Spectrophotometer UV-(1800) was used for all spectrophotometric measurements.

CAT activity was measured according to Aebi [22]. The rate of decomposition of H_2O_2 was measured spectrophotometrically from change in absorbance at 240 nm. Activity of catalase was expressed as μmol H_2O_2/mg protein.

GR activity was determined by following the oxidation of NADPH at 340 nm (extinction coefficient 6.22 mM^{-1} cm^{-1}) as described by Carlberg and Mannervik [23]. GR activity was expressed as μmol NADPH oxidized/min/mg protein.

The activity of GPx was measured using a coupled enzyme assay system linked with GR as described by Lawrence and Burk [24]. Enzyme activity was calculated as μmol of NADPH oxidized per min per mg protein using the molar extinction coefficient for NADPH at 340 nm of 6.22 $mM^{-1}cm^{-1}$.

GST activity using 1-chloro-2,4-dinitrobenzene as substrate was assayed spectrophotometrically as described by Habig and Jakoby [25]. Specific activity was expressed as μmol conjugate formed/min/mg protein using a molar extinction coefficient of 9.6 $mM^{-1}cm^{-1}$.

Total SOD activity was measured by its ability to increase the effect of riboflavin sensitized photo-oxidation of *o*-dianisidine, according to Mylroie et al.[26].

Tissue MPO levels were measured according to Hillegass et al.[27]. 1 U of activity was defined as change in absorbance of 1.0 per minute at 25°C. Results were expressed as units of MPO per gram of protein of supernatant as determined by method of Lowry.

The *p*-nitrophenylacetate esterase activity of CA was measured by the method of Verpoorte et al.[28]. One unit of enzyme activity was expressed as μmol nitrophenol formed per minute at 0°C using a molar extinction coefficient of 5 x 10^{-3}.

Protein carbonyl content (PCC) was assayed by the modification of the procedure described by Levine et al.[29] and Reznick and Packer [30] using dinitrophenylhydrazine (DNPH) dissolved in HCl, accompanied by blanks in HCl alone. Results were expressed as nmol of protein carbonyl per mg of protein using a molar extinction coefficient of 22.000 $M^{-1}cm^{-1}$ for DNPH.

Tissue protein levels were measured by the method of Lowry et al.[31] using bovine serum albumin as a standard.

Statistical Analysis

Biochemical results were evaluated using an unpaired *t*-test and ANOVA variance analysis using the NCSS statistical computer package. The values were expressed as mean ± SD. Analysis between control and experimental groups was performed using the Mann-Whitney test. $P<0.05$ was considered as significant.

RESULTS

A significant increase was observed in kidney tissue CAT activity in STZ-diabetic rats ($P<0.001$). Zinc sulfate treatment caused a significant decrease ($P<0.005$) in the activity of this enzyme. The difference between the groups was significant ($P_{ANOVA}= 0.0001$; *Table 1*).

GR activity was significantly higher in STZ-diabetic rats kidney tissue compared to control group ($P<0.05$). The decrease in GR activity in the Zn treated group was not significant. The difference between the groups was significant ($P_{ANOVA}= 0.01$; *Table 1*).

Zn treatment to normal animals in the control group caused a significant rise in GPx activity in rat kidney tissue ($P<0.05$). Induction of diabetes rised significantly GPx

activity compared to the control group (P<0.001) and Zn treatment lowered significantly (P<0.05) the enzyme activity. The difference between the groups was significant (P_{ANOVA}= 0.0001; *Table 1*).

A significant increase was also seen in GST activity levels in the diabetic group *versus* the control group (P<0.005) whereas the levels were lowered almost to control levels (P<0.05) in the Zn treated group. The difference between the groups was significant (P_{ANOVA}= 0.0001; *Table 1*).

SOD activity was also significantly increased in the kidney tissue of diabetic rats (P<0.05) and though insignificant, a decrease was observed after zinc sulfate administration. The difference between the groups was significant (P_{ANOVA}= 0.009; *Table 1*).

Kidney tissue MPO activity which was significantly increased (P<0.001) by the induction of diabetes, was lowered by treatment with zinc sulfate (P<0.005). The difference between the groups was significant (P_{ANOVA}= 0.0001; *Table 2*).

STZ-induced diabetes provoked a significant increase (P<0.05) in kidney tissue CA activity, the diabetic group which have received zinc sulfate showed significant decrease (P<0.05) in these values compared to the diabetic group. The difference between the groups was significant (P_{ANOVA}= 0.003; *Table 2*).

An insignificant increase of PCC was observed in the kidney tissue of diabetic rats. This value was decreased in the diabetic group treated with zinc (P_{ANOVA}= 0.062; *Table 2*).

In summary, Zn administration made an unsignificant rise in the antioxidant enzyme levels in the control + Zn group. In STZ-diabetic group all enzyme activities were raised significantly and it was observed that Zn treatment reverses significantly the oxidative damage caused by diabetes.

DISCUSSION

In a previous study, significant hypoglycemic effect was seen between the fasting blood glucose levels of the diabetic group and diabetic + Zn group [10]. This suggests that Zn supplementation stimulates glucose metabolism and is in agreement with literature reporting protective effects of Zn on blood glucose levels [32,33].

The role of Zn in modulating oxidative stress has been recognized, thus Zn could be a physiological constituent of the antioxidant defense system. Zn deficiency has been demonstrated to trigger oxidative stress and oxidant-mediated damage to cell components [34]. Combined supplementation of chromium and Zn in people with type 2 diabetes mellitus had potential beneficial effects with

Table 1. *The activities of antioxidant enzymes, catalase (CAT), glutathione reductase (GR), glutathione peroxidase (GPx), glutathione-S-transferase (GST) and superoxide dismutase (SOD) in the kidney tissues of all groups*

Tablo 1. *Tüm grupların böbrek dokularında, katalaz (CAT), glutatyon redüktaz (GR), glutatyon peroksidaz (GPx), glutatyon-S-transferaz (GST) ve superoksit dismutaz (SOD) antioksidan enzimlerinin aktiviteleri*

Group	CAT (U/mg protein)	GR (U/mg protein)	GPx (U/mg protein)	GST (U/mg protein)	SOD (U/mg protein)
Control	27.65±7.02	5.46±2.6	99±15.11	45.67±12.13	0.071±0.01
Control+Zn	38.81±12.81	8.52±3.64	119.34±3.46***	51.32±9.33	0.141±0.05
Diabetic	72.17±3.97*	17.43±4.52***	491.7±84.85*	123.16±19.17*****	0.263±0.08***
Diabetic+Zn	56.97±2.17**	12.77±5.48	319.4±52.35****	73.32±22.02****	0.147±4.92
P_{ANOVA}	0.0001	0.01	0.0001	0.0001	0.009

*Values are given as Mean ± S.D.; * P< 0.001 compared with control; ** P<0.005 compared with diabetic; *** P<0.05 compared with control; **** P<0.05 compared with diabetic; ***** P<0.005 compared with control*

Table 2. *The activities of myeloperoxidase (MPO) and carbonic anhydrase (CA) and the levels of protein carbonyl contents (PCC) in the kidney tissues of all groups*

Tablo 2. *Tüm grupların böbrek dokularında, miyeloperoksidaz (MPO) ve karbonik anhidraz (CA) aktiviteleri ve protein karbonil miktarları (PCC)*

Group	MPO (U/g protein)	CA (U/mg protein)	PCC (nmol PC/mg protein)
Control	4.28±0.97	5.98±0.36	1.22±0.75
Control+Zn	5.10±0.76	12.59±4.67	1.95±0.89
Diabetic	10.72±1.14*	24.07±8.32***	6.49±4.84
Diabetic+Zn	7.55±1.5**	11.80±3.84****	3.02±1.58
P_{ANOVA}	0.0001	0.003	0.062

*Values are given as Mean ± S.D.; * P< 0.001 compared with control; ** P< 0.005 compared with diabetic; *** P<0.05 compared with control; **** P<0.05 compared with diabetic*

significant reduction of plasma thiobarbituric acid reactive substances (TBARS) [26]. Zn supplementation of diabetic patients ameliorated initially increased parameters of lipid peroxidation in both forms of diabetes mellitus [7,35].

In some studies, reduced activities of SOD and CAT in tissues of diabetic animals have been related to the increased production of ROS [36]. A decrease in SOD activity by increased intake of Zn has also been reported [37]. On the contrary, in agreement with the findings of the present study, the activity of CAT was increased in diabetic animals tissues [38]. Similarily, increase in GR and GPx activities in the heart tissue of diabetic rabbits has been reported to be an efficacious defense against oxidative stress [39]. GPx has also been shown to be an important adaptive response to conditions of increased peroxidative stress [40]. GSTs belong to a group of multigene and multifunctional detoxification enzymes and an important condition affecting GST expression is known to be oxidative stress [41].

Zn is an inhibitor of the enzymes NADPH oxidases which catalyzes the production of O_2^- from oxygen by using NADPH as the electron donor. The dismutation of O_2^- to H_2O_2 is catalyzed by another enzyme, SOD which contains copper and Zn [42]. It was reported that Zn deficiency induce lipid peroxidation, increase free radical generation and decrease hepatic CuZn-SOD activity in exercised mice [43]. An additional antioxidative mechanism by which Zn may be functioning was proposed by Prasad et al.[42] in a study in which they have shown that Zn negatively regulates gene expression of inflammatory cytokines such as TNFa and IL-1β.

MPO is a heme peroxidase released by polimorphnuclear neutrophils which catalyzes the formation of numerous ROS and thus has strong proinflammatory and pro-oxidative properties [44]. This enzyme changes hydrogen peroxide to cytotoxic compound, hypochloric acid [45].

In the present study, the fact that in diabetic kidney CAT, GR, GPx, GST, SOD and MPO activities were increased, leads to the consideration that the expression of antioxidant defense enzymes augmented due to increased free radical production. Administration of Zn have reversed the effect showing that antioxidant defense is no more needed and thus confirming the beneficial effect of zinc sulfate on the oxidation provoked by diabetes induction.

In a previous study undertaken on the stomach tissue of STZ-diabetic rat models, it was demonstrated that Zn supplementation shows a protective effect on impaired oxidative stress parameters such as LPO, GSH, NEG levels [46]. The increase in antioxidant enzymes in the kidney tissue of STZ-diabetic rats and the beneficial effect of Zn determined in the present study, are in agreement with these findings. In a recent study, pretreatment with zinc was found effective in preventing indomethacin-induced renal damage in rats, by ameliorating oxidative stress [47].

It has been reported that STZ-induced diabetes mellitus resulted in a reduction in concentration of CA-III, which is a key enzyme in acid-base regulation of the kidney, in some tissues of rats [48]. As CA is known to be present in rat kidney mitochondria [49] and be involved in rat renal glucose synthesis as well [50], we have investigated the effect of Zn supplementation on this enzyme in STZ-diabetic rat models. The significant change in CA levels after Zn administration in the control + Zn group, is probably due to the activator effect of Zn on CA.

Oxidative stress modifies body proteins, carbohydrates and lipids with generation of reactive carbonyl compounds. It has been hypothesized that "carbonyl stress" may be a causative factor for lipid peroxidation and chronic complications of type 2 diabetes mellitus, like nephropathy, neuropathy and coronary artery disease [51]. It was reported that PCC were significantly increased in diabetic kidney tissue homogenates [52]. In accordance, in the present study an increase (though statistically insignificant) in PCC levels was observed in the diabetic group and Zn treatment seems to be effective in lowering these levels similarily to antioxidant enzymes activities.

Considering the beneficial effects of Zn supplementation on glycemic control in type 1 and type 2 diabetic animals and humans, its insulinomimetic effects, the zinc-mediated protection of β cells from damage by immune cells and cytokines and its antioxdant effect demonstrated in the present study, Zn might be regarded as a possible new candidate molecule for diabetes prevention and therapy, especially for type 2 diabetic patients. However, the Zn dose administered has to be determined individually and the Zn status should be controlled in order to prevent adverse effects based on inappropriately high Zn dosage [11].

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