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The effects of copper supplement on zinc status, enzymes of zinc activities and antioxidant status in alloxan-induced diabetic rats fed on zinc over-dose diet

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The aim of this study was to investigate the effect of copper supplementation on over-dose zinc in experimental diabetes. Male alloxan-induced diabetic Wistar albino rats of 12 weeks of age were divided into three groups. The first group received a diet containing 54 mg zinc/kg (control group), the second group received a diet containing 231 mg zinc/kg (Zn group), and the third group received a diet containing 231 mg zinc/kg supplemented with copper (30 mg/kg diet) (Zn+Cu group). Body weight gain of all rats was recorded regularly over a period of three weeks. On day 21, after overnight fasting, animals were sacrificed and blood glucose, zinc concentration, and amylase, aldolase, lactate dehydrogenase and alkaline phosphatase activities and parameter antioxidants were determined on tissues and serum samples. Body weight gain of copper supplementation diabetic animals at the end of three weeks of dietary manipulation was significantly lower with a percentage of 41.09% than that of zinc over dose diabetic animals. The administration of copper significantly altered blood glucose with a percentage of 22.98%, serum and tissues zinc concentration ($P < 0.01$) and all enzymes zinc dependants in animals. Copper added significantly increased glutathione (GSH) concentration ($P < 0.05$) and glutathione peroxydase (GPx) activity ($P < 0.01$) in rats. In contrast, liver malondialdehyde (MDA) and testis glutathione S transferase (GST) activity levels were lower with a percentage of 13.52 and 29.78%, respectively. There is no statistically change in liver GST activity in (Zn+Cu) group. It was concluded that supplementation of copper diet have significantly reduced the zinc status, disrupt the activity of zinc-dependent enzymes and altered in the chemical and oxidative parameters in diabetes.

Key words: Diabetic rats, alloxan, zinc status, copper, antioxydants.

INTRODUCTION

Life would not be possible without a large number of 'trace' elements, each serving critical roles in metabolism and function. Trace elements are necessary for normal function and are therefore associated with morbid

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deficiency states. They are also commonly toxic when present in excess (Huffman and O'Halloran, 2001). Zinc and copper are two of the most metabolically active and intensively investigated trace metal nutrients. The established biochemical role of zinc is as a component of more than 300 metalloenzyme (Atukorala and Waidyanatha, 1987) by participating in their structure or in their catalytic and regulatory actions. It is a structural ion of biological membranes and closely related to protein synthesis. The concept of zinc fingers explains the role of zinc in gene expression and endocrine function, and mechanisms of action of zinc involve the effects of the metal on DNA synthesis, RNA synthesis, and cell division. Zinc also interacts with important hormones involved in bone growth such as somatomedin-c, osteocalcin, testosterone, thyroid hormones, and insulin (Maria et al., 2002).

Oxidative stress is caused by a relative overload of oxidants, reactive oxygen species zinc-containing enzymes participate in many components of macronutrient metabolism, particularly in cell replication. In addition, zinc-containing enzymes such as carbonic anhydrase and lactate dehydrogenase are involved in exercise metabolism while superoxide dismutase protects against free radical damage (Parvaneh et al., 2009). There are characteristic and specialized systems of zinc absorption, transport and excretion in human organism. The synergic and antagonist effects of minerals with zinc influence the quantum of zinc in a certain organ (Richard et al., 2001). Cu is also an essential micronutrient required by all life forms. Cu is a transition metal and hence involved in a variety of biological processes, embryonic development, mitochondrial respiration, regulation of hemoglobin levels as well as hepatocyte, neuronal functions and free radical detoxification.

Cu is vital for normal healthy functioning of organisms (Krupanidhi et al., 2008). Dietary copper, absorbed in the stomach and upper intestinal tract, reaches liver as a complex with serum proteins, albumin or transcuperin or the amino acid histidine. Liver is the major store house for intracellular copper need to be maintained in a complex state so as to prevent the oxidative damage caused by free copper to DNA, proteins and membrane components (Krupanidhi et al., 2008). Because copper is a redox metal, unfettered copper is a potential oxidant (or reductant) of cellular proteins, lipids and nucleic acids. This property mandates copper to be in a bound form and not a free ion in blood, extracellular fluids or cytosol. Of its two major valencies, (Cu (I) and Cu (II)), Cu (I) behaves as a progenitor of free radicals. Metal ion, Cu (I) also has the potential to antagonize Zn (II) (Huffman and O'Halloran, 2001).

Oxidative damage due to free radicals is associated with vascular disease in people with type 1 and those with type 2 diabetes mellitus (DM). There are several potential sources of increased free radical production in diabetes including auto-oxidation of plasma glucose, activation of leucocytes and increased transition metal bioavailability. The radical-scavenging antioxidant activity of the serum of people with either type 1 or 2 DM is lower than that of age-matched controls. This may be attributed to lower blood orate, vitamin C or vitamin E or other factors including the trace elements. Several reports underlie the key role of micronutrient status in patients with type 1 or 2 DM. Correction of Zn deficiency in patients with type 1 DM by zinc over dose also leads to decreased lipid peroxidation and improvements in glucose homeostasis. Therefore, Zn act in normalizing glycemia and is postulated to function as antioxidants, a restored Zn status in people with type 2 DM may counteract the deleterious effects of oxidative stress and help to prevent complications associated with diabetes (Richard et al., 2001). Therefore, the aim of this study was to examine the effects of dietary copper supplementation and its effect on diabetic pathology observed in zinc over dose rats by evaluating body weight gain, zinc status, some zinc dependant enzyme activities and antioxidants status in alloxan induced diabetic rats.

MATERIALS AND METHODS

Animals and diet

Male albino (Wistar) rats of two to three months of age, weighing 220 to 300 gm were housed in standards cages. Humidity and temperature were controlled with a 12 h light/dark cycle. Animals were left to feed and were given water *ad libitum*. After one week, rats were injected intraperitoneally with a freshly prepared alloxan monohydrate solution (Alloxan; Sigma, UK) at a dose of 150 mg/kg of body weight (Awadallah and Dessoukey, 1977) to induce diabetes. Seven days after alloxan administration serum glucose concentration was measured. All alloxan-treated animals in which glucose concentration in the serum was lower than 11 mmol/L (1.98 g/L) were excluded from the experiment. The animals were divided into three groups (10 rats per group) after the stabilization of diabetes. The first group received a diet containing a 54 mg zinc/kg diet (adequate zinc, group control), the second group received a diet containing a 231 mg $ZnSO_4.7H_2O$/kg diet (Dorota et al., 2011) (zinc over-dose, Zn group) and the third group received a diet containing a 231 mg $ZnSO_4.7H_2O$/kg diet supplemented with copper (30 mg $CuSO_4.5H_2O$/kg diet) (Bremner et al., 1976) (Zn+Cu group). The composition of the diet was similar to that described previously by Southon et al., (1984). Rats were maintained on the appropriate experimental diet *ad libitum* for 20 days. Body weight gain was monitored weekly and at the time of sacrifice (after 1, 2 and 3 weeks of disease). Animals were fasted overnight on day 20, and on day 21 given access to food for two periods of 1 h between 11.00 to 12.00 h and 17.00 to 18.00 h so that time of feeding on day before they were sacrificed was similar for all groups.

Rats were then sacrificed between 11.00 and 12.30 h on day 22. One animal from each group was sacrificed approximately at the same time by exsanguinations from the heart whilst under diethyl-ether anaesthesia. Blood was transferred into ice cold centrifuge tubes and a portion was taken for whole-blood glucose analysis which was performed immediately after exsanguinations. The

Table 1. Body weight gain, serum zinc and tissues zinc concentrations of diabetic rats fed adequate zinc diet (control), zinc over dose diet (Zn) and zinc over dose diet supplemented with copper (Zn+Cu) at the end of experimental period.

Parameter	Experimental groups		
	Control (n=10)	Zn (n=10)	Zn+Cu (n=10)
	Mean SEM	Mean SEM	Mean SEM
Body weight gain (g/day)	3.99±0.51	4.38±0.44	2.58±0.88**
Zinc serum(µg/100ml)	90.30±3.40	147.12±9.21	93.20±12.0**
Zinc liver (µg/g dry weight)	28.59±1.18	35.48±2.40	29.50±3.43*
Zinc kidney(µg/g dry weight)	35.19±3.14	45.98±7.72	40.09±6.35*
Zinc pancreatic(µg/g dry weight)	59.82±5.21	76.32±8.52	61.30±3.82**
Zinc testis(µg/g dry weight)	81.70±4.05	170.50±7.08	89.92±5.83***

*(P < 0.05) ; **(P < 0.01) ; ***(P < 0.001).

remaining blood was centrifuged for 10 min at 3,000 revolutions/min and the serum was utilized for serum zinc, amylase, aldolase, lactate dehydrogenase and alkaline phosphatase assays. The liver, kidney, pancreas and testis of each animal was removed, washed with ice-cold physiological saline solution, dried and processed for biochemical measurements. Homogenates were prepared on ice in the ratio of 4 g tissue in 16 ml of phosphate buffer, pH 7.5, containing 1 mmol/l Na_2EDTA. For each sample, 10 µl of 500 mmol/l BHT in acetonitrile was added to prevent formation of new peroxides during the assay. The homogenates were centrifuged at 20,000 × g for 15 min at 4°C and frozen at -70°C until analysis.

Analytical methods

Blood glucose was estimated by the glucose oxidase method using commercial test kit for glucose. Serum amylase, aldolase, lactate dehydrogenase and alkaline phosphatase activity were determined using commercial test kits for amylase (Ying Foo 1998), aldolase (Feissli 1966), lactate dehydrogenase (Pesce and Kaplan, 1984) and alkaline phosphatase (Bowers and Macomb, 1966). Dried liver, pancreas, testis and kidney were heated in silica crucibles at 480°C for 48 h and the ash taken up in hot Ultrex nitric acid (11.7 M) for Zn analysis by atomic absorption spectrophotometer (SHIMADZU AA-6200) China (Kechrid and Bouzerna, 2001). The accuracy of zinc recovery was checked using standard reference materials; bovine liver and wheat flour. These standards were prepared and analyzed in similar conditions to the test items to assess recovery. The recovery of zinc in the standard reference material exceeded 96%. Zinc in serum was analysed after a twenty-fold dilution of the serum by flame atomic absorption spectrophotometer (SHIMADZU AA-6200). Zinc standards were prepared from a 1 mg/ml zinc nitrate standard solution (BDH) using 5% glycerol to approximate the viscosity characteristics, and to avoid zinc contamination from exogenous sources. All tubes were soaked in HNO_3 (10% v/v) for 16 h and rinsed with double distilled water. Glutathione (GSH) concentration was measured utilizing the method described by Weckbercker and Cory (1988). Malondialdehyde (MDA) concentration was determined by spectrophotometry method of Okhawa et al. (1979). GSH-Px (EC 1.11.1.6) activity was measured spectrophotometrically at 340 nm by the Pinto and Brtley method (1969), as regards the activity glutathione S transferase, it was determined by the Habig et al. (1974) method. The reported data are the means of measurements and their standard error of mean (SEM) values. For statistical evaluation, the Student's t-test and P < 0.05 was considered the limit for the statistical significance.

RESULTS

Table 1 summarizes the body weight gain, serum zinc, liver zinc, kidney zinc, pancreatic zinc and testis zinc concentrations of the animal groups studied. Body weight gain of diabetic zinc over dose animals (Zn) at the end of three weeks of dietary manipulation was significantly higher than those of adequate zinc diabetic rats (AZ) by 4.38 ± 0.44 to 3.99 ± 0.51. Compared with adequate zinc diabetic group, zinc over dose diabetic group had high serum zinc, liver zinc, kidney zinc, and pancreatic zinc and testis zinc levels. Copper significantly decreased the body weight gain (P < 0.01), serum zinc (P < 0.01), liver zinc and kidney zinc (P < 0.05), pancreatic zinc (P < 0.01) and testis zinc (P < 0.001) levels of zinc over dose diabetic rats (Table 2). Blood glucose was lower of zinc over dose diabetic rats than those of zinc adequate diabetic animals by 1.61 ± 0.01 to 2.08 ± 1.15. However, serum amylase, aldolase, lactate dehydrogenase and alkaline phosphatase activity of diabetic zinc over dose diabetic group (Zn) were higher than those of zinc adequate diabetic group (control). Supplementation of the diet of zinc over dose alloxan diabetic rats with copper significantly reduced amylase (P < 0.001), aldolase (P < 0.05), lactate dehydrogenase (P < 0.01) and alkaline phosphatase (P < 0.001) activities (Table 2).

The liver malondialdehyde (MDA), liver glutathione (GSH) and testis GSH levels in zinc over dose diabetic group (Zn) were increased compared to the adequate zinc diabetic group. Supplemented with copper showed a significantly reduction of liver MDA level (P < 0.001) and elevated liver GSH (P < 0.05) and testis GSH levels (P < 0.05) (Table 3). The liver glutathione S transferase (GST) and testis GST activities in zinc over dose diabetic group

Table 2. Mean blood glucose and enzymes of zinc activities of diabetic rats fed adequate zinc diet (control), zinc over dose diet (Zn) and zinc over dose diet supplemented with copper (Zn+Cu) at the end of experimental period Dry

Parameter	Experimental groups		
	Control (n=10) Mean SEM	Zn (n=10) Mean SEM	Zn+Cu (n=10) Mean SEM
Blood glucose (g/l)	2.08±1.15	1.61±0.01	1.98±0.01**
Serum amylase (U/l)	623.21±71.22	765±14.2	541.8±24.7***
Serum alkaline phosphatase (U/l)	212.31±12.90	259.8±31.9	231.8±167***
Serum lactate dehydrogenase (U/l)	1132.60±47.16	1262.3±63.4	1134±365**
Serum aldolase (U/l)	6.05±0.29	7.8±0.1	6.8±0.15*

*(P < 0.05) ; **(P < 0.01) ; ***(P < 0.001)

Table 3. Antioxidant enzyme activity levels and concentrations of GSH and MDA in liver and testis in the diabetic rats fed adequate zinc diet (control), zinc over dose diet (Zn) and zinc over dose diet supplemented with copper (Zn+Cu) at the end of experimental period.

Parameter	Experimental groups		
	Control (n=10) Mean SEM	Zn (n=10) Mean SEM	Zn+Cu (n=10) Mean SEM
Liver GSH (nM/mg protein)	32.92±1.57	45.92±2.74	50.53±5.26*
Testis GSH (nM/mg protein)	24.58±1.30	34.01±0.90	35.6±1.70*
Liver MDA (nM/ mg protein)	182.41±34.2	212.9±69.5	184.1±54.6***
Liver GST (µM/g protein)	0.38±0.03	0.44±0.08	0.42±0.16
Testis GST (µM/g protein)	0.41±0.07	0.47±0.03	0.33±0.08***
Liver GPx (mU/g protein)	51.4±3.60	38.18±2.93	41.5 6±2.60**
Testis GPx (mU/g protein)	33.4±2.60	24.7±1.12	37.15±0.05***

*(P < 0.05); **(P < 0.01); ***(P < 0.001).

(Zn) were increased, compared to the adequate zinc diabetic group. However, the liver glutathione peroxydase (GPx) and testis GPx activities of zinc over dose animals was lower than those of adequate zinc animals. Addition of copper significantly reduced testis GST by 0.33 ± 0.08 to 0.47 ± 0.03 (P < 0.01) , elevated liver GPx, testis GPx activities by 41.5 6 ± 2.60 to 38.18 ± 2.93 and 37.15 ± 0.05 to 24.7 ± 1.12 (P < 0.001), respectively and non significantly variation liver GST activity (Table 3).

DISCUSSION

In our study, body weight gain was affected by copper by 2.58 ± 0.88 to 4.38 ± 0.44 (P < 0.01). It is demonstrated that high Cu in diet have damaged duodenal villi, therefore, have impact on nutrient absorption, depress food intake resulting in poor growth performance and decrease weight body (Rahman et al., 2001). Zinc concentrations in serum and tissues were assayed to confirm zinc over dose diet .The zinc absorption have strong connection with the copper absorption, because of their competition for the carrier (Dimitrova et al., 2010). It is suggested thus that copper was bounded to zinc and it resulted in depletion of zinc free level as confirmed by the result of low zinc concentration, which was found in serum. This growth retardation was due in part to a decrease in appetite and impaired protein synthesis (Kechrid et al., 2007a). Serum zinc, liver, pancreatic, kidney and testis zinc concentrations in rats fed zinc over dose diet were higher than that of adequate zinc group. Supplementation with copper (30 mg/kg) significantly reduced body zinc status. These findings indicated the effect of copper on decreased bioavailability of the zinc which is in agreement with investigation of Willis et al. (2005).

In the current study, when the time of feeding was strictly controlled and the amount of food eaten by each animal before an overnight fast was known to be similar, the mean fasting blood glucose concentration in rats fed

zinc over dose diet supplemented with copper were found to be higher than that of rats fed zinc over dose diet by 1.98 ± 0.01 to 1.61 ± 0.01 ($P < 0.01$). This suggests that copper caused reduction in level of free zinc (Dimitrova et al., 2010). Thus it exacerbated the ability of diabetic rats to utilize glucose. Results from previous studies showed an increased blood glucose concentration after intravenous injection with glucose in rats fed on zinc over dose diet (Kechrid et al., 2007b), suggesting a relation between carbohydrate utilization and the zinc status. A decrease in the plasma glucose concentration in Cu-supplemented may be due to decreased zinc intake. The decrease in Zn dependent enzymes activities of serum (amylase, aldolase, lactate dehydrogenase and alkaline phosphatase) in rats given over dose zinc diet supplemented with copper may be attributed to the decrease in serum zinc.

Zinc is essential for the activity of amylase (Savchenko et al., 2002), aldolase (Matthias and Georg, 1996), lactate dehydrogenase (Parvaneh et al., 2009), and alkaline phosphatase (Jamshid et al., 2011). It serves as one or more structural, regulatory or catalytic functions (Kechrid and Kenouz, 2003). In general, the present study indicated that some symptoms and signs associated with zinc deficiency and decrease metallo-enzyme in diabetic rats can be stimulated by supplementation with copper. The increase of GSH, MDA concentration and GST activity in animals fed Zn confirms an efficacious defense of the zinc against oxidative stress under diabetic conditions (Mariani et al., 2008). Zinc is also necessary to stimulate defense against reactive species oxygen and H_2O_2 that induce apoptosis (Ani et al., 2007). Supplementation with copper decrease MDA concentration and increase GSH, liver GPx activity by 184.1 ± 54.6 to 212.9 ± 69.5 ($P < 0.001$), 50.53 ± 5.26 to 45.92 ± 2.74 concentration ($P < 0.05$) and 41.5 6 ± 2.60 to 38.18 ± 2.93 ($P < 0.01$), respectively.

Liver GSH S-transferase activity in rats fed Zn+Cu was the same as in rats fed Zn, indicating this activity is unaffected by copper supplement. This result confirms the report of Stephen et al. (1982) who observed GSH S-transferase activity in liver unaffected by copper in rats. However, the decreased liver concentration of MDA in (Zn + Cu) fed ($P < 0.001$) is probably a result of decreased zinc by copper supplemented. Copper-supplemented cells accumulate glutathione through a decrease in glutathione efflux and not by an increase in GSH synthesis.

In contrast, elevated GSH levels tissues from rats exposed to zinc were caused by increases in GSH synthesis (Jonathan et al., 1989). Copper is essential for superoxide dismutase (SOD) activity. Different studies have confirmed that the production of hydrogen peroxide (H_2O_2) under the action of SOD (Joanny and Menvielle, 2005). Peroxide has been shown to react with Cu to form highly toxic hydroxyl radicals (Jonathan et al., 1989). An elevation in the activity of hydrogen peroxide metabolizing GSH peroxidase is believed to be an indication of increased cellular hydrogen peroxide flux. It has been suggested that increased GSH peroxidase activity may cause cells to develop higher levels of GSH (Jonathan et al., 1989).

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