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Advanced glycation end products and antioxidant status in nondiabetic and streptozotocin induced diabetic rats: effects of copper treatment

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Abstract The effects of Cu(II) supplementation on glycemic parameters, advanced glycation end products (AGEs), antioxidant status (glutathione; GSH and total antioxidant capacity; TAOC) and lipid peroxidative damage (thiobarbituric acid-reactive substances, TBARS) were investigated in streptozotocin (STZ) induced diabetic rats. The study was carried out on Wistar albino rats grouped as control ($n = 10$), $CuCl_2$ treated ($n = 9$), STZ ($n = 10$) and STZ,$CuCl_2$ treated ($n = 9$). STZ was administered intraperitoneally at a single dose of 65 mg/kg and $CuCl_2$, 4 mg copper/kg, subcutaneously, every 2 days for 60 days. At the end of this period, glucose(mg/dl), Cu(μg/dl), TBARS(μmol/l), TAOC(mmol/l) were measured in plasma, GSH(mg/gHb) in erythrocytes and glycated hemoglobin (GHb)(%) in blood. Plasma AGE-peptides(%) were measured by HPLC flow system with spectrofluorimetric and spectrophotometric detectors connected on-line. Data were analyzed by the nonparametric Kruskal–Wallis and Mann–Whitney U test. In the STZ group glucose, GHb and AGE-peptide levels were all significantly higher than the control group ($P < 0.01$, $P < 0.05$, and $P < 0.01$, respectively). $CuCl_2$ treated group had significantly lower glucose but significantly higher GHb, TAOC and TBARS levels than the control group ($P < 0.05$, $P < 0.001$, $P < 0.05$ and $P < 0.001$, respectively). STZ,$CuCl_2$ treated group had significantly higher GHb, TAOC and TBARS levels compared with the control group ($P < 0.001$, $P < 0.05$ and $P < 0.05$, respectively); but only TAOC level was significantly higher than the STZ group ($P < 0.01$). This experimental study provides evidence that copper intake increases total antioxidant capacity in both nondiabetic and diabetic states. However despite the potentiated antioxidant defence, lipid peroxidation and glycation enhancing effects of $CuCl_2$ are evident under nondiabetic conditions.

Keywords STZ · Diabetes mellitus · Advanced glycation end products · Glycation · Copper

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Introduction

Increased oxidative stress promotes protein glycation (Selvaraj et al. 2002). As free Cu ions are highly redox active and thus might contribute to oxidative tissue damage, in vivo availability of catalytic Cu is usually restricted to a great extent (Cooper et al. 2005). However, glycation due to chronic hyperglycemia has been reported to damage the Cu-binding properties of

ceruloplasmin and albumin, the main Cu-binding proteins in plasma (Linder 1991; Islam et al. 1995; Argirova and Ortwerth 2003). On one hand, glycation lowers the capacity of these proteins to bind metal ions in strong complexes (Argirova and Ortwerth 2003). On the other hand, the redox active glycochelates are sites for reactive oxygen species (ROS) production (Eaton and Qian 2002). Thus, for restriction of glycoxidation related pathologies in diabetes mellitus, chelation of Cu-ions by clinically acceptable compounds is suggested (Cooper et al. 2005).

However, it is also known that copper sulfate treatment started prior to streptozotocin (STZ) injection is beneficial in diabetic mice. It causes preservation of β-cell function (Sitasawad et al. 2001). As yet, it is not known whether Cu intake in the diabetic condition is beneficial or not. This study aims to investigate and compare the effects of Cu treatment on glycemic parameters, advanced glycation end products (AGEs), antioxidant status parameters namely glutathione (GSH) and total antioxidant capacity (TAOC) and lipid peroxidative damage marker, thiobarbituric acid-reactive substances (TBARS) in STZ induced diabetic rats with respect to nondiabetic controls.

Materials and methods

Animal treatment and diabetes induction

Male, young adult Wistar albino rats (2.5–3 months, $n = 38$ weighing 180–200 g) were used in this study. The rats cared for in accordance with the Guide for the Care and Use of Laboratory Animals (Committee on Care and Use of Laboratory Animals 1985) received standard chow diet (20–30 g/rat/day) and water ad libitum during the experimental period of 60 days.

The following experimental groups were designed: Control group (control, $n = 10$); Cu treated group ($CuCl_2$, $n = 9$); STZ administered group (STZ, $n = 10$) and STZ administered and Cu treated group (STZ,$CuCl_2$, $n = 9$).

Diabetes was induced by intraperitoneal injection of a single dose of streptozotocin (STZ) (65 mg/kg body weight) (Sigma-Aldrich) freshly dissolved in citrate buffer (pH = 4.5) after an overnight fast. Control rats were injected citrate buffer alone. Three days after STZ injection, development of diabetes was confirmed by the presence of hyperglycemia and glycosuria. Diabetic rats manifested polyphagia and glycosuria throughout the experimentation period. Three days after STZ injection, the rats, in groups $CuCl_2$ treated and STZ,$CuCl_2$ treated, started receiving copper subcutaneously at a dose of 4 mg/kg body weight, on every alternate day for 60 days. Copper solutions were prepared immediately before use. At the end of this period, the rats were weighed and killed by decapitation under ether anesthesia (50 mg/kg) after an 8 h fast. Heparinized blood samples were obtained by cardiac puncture. A small aliquot was refrigerated for GHb assay on the following day. After centrifugation at $2{,}500\times g$ for 5 min, plasma glucose and erythrocyte GSH analyses were done immediately. The samples were stored at -80°C until analysis for TBARS, TAOC, AGE-peptides and Cu.

Biochemical analysis

Assay of glucose and GHb

Glucose was determined using GOD-PAP (Randox) and GHb, by boronic affinity chromatography (Sigma) kits.

Assay of AGE

Fluorescent AGE-peptides present in plasma were measured by spectrofluorimetry (λ_{ex}: 247 nm, λ_{em}: 440 nm) and the related peptides by spectrophotometry at 280 nm, in a HPLC flow system with spectrofluorimetric and spectrophotometric detectors connected on-line (Wrobel et al. 1997; Forbes et al. 2004). Briefly, 20 µl plasma was added to 480 µl of 0.15 mmol/l trichloroacetic acid and 100 µl chloroform. Samples were shaken vigorously, centrifuged, and 20 µl of the aqueous layer was then injected into the flow system, consisting a Hewlett Packard high pressure pump (Series 1050 high-performance liquid chromatograph), with integrated sample injector (loop 20 µl) and multiple wavelength spectrophotometric detector (Series 1100) and spectrofluorimetric detector (HP 1046A). Chemstation (Series 1100) was used for data processing. Samples were run in triplicate.

The assay was calibrated against AGE-peptide obtained from enzymatic hydrolysis of AGE-BSA (10 g/l). Albumin was incubated for 60 days with glucose to prepare AGE-albumin. Albumin was also

incubated in glucose-free medium under similar conditions. AGE-albumin and albumin were hydrolysed by proteinase K to prepare AGE-peptides and peptide calibrators, respectively. The AGE content of the AGE-peptide calibrator was accepted as 100% and by dilution with the peptide calibrator, different concentrations of the calibrator was prepared.

Assay of copper

Cu in plasma was determined by flameless atomic absorption spectrophotometry (atomic absorption spectrophotometer, model AA-6800; Shimadzu). The standard, diluted plasma sample is delivered onto a pyroltically coated carbon tube. A temperature program was initiated, and the instrument in turn dries, ashes, and then atomizes the sample. The vaporized neutral atoms absorb light from a copper hollow cathode lamp set at 324.7 nm. The absorbance was directly related to the concentration of copper, expressed in micrograms per deciliter in sample Alcock (1987) and Shimatzu Corp (1998).

Assay of thiobarbituric acid-reactive substances (TBARS)

Lipid peroxidation end product was determined as TBARS according to a modification of the method of Buege and Aust (1978). One volume of plasma was mixed thoroughly with two volumes of a stock solution of 15% w/v trichloroacetic acid, 0.375% w/v thiobarbituric acid, and 0.25 mol/l hydrochloric acid. The mixture was heated for 30 min in a boiling water bath. After cooling, the flocculent precipitate was removed by centrifugation at 1000 g for 10 min. The absorbance of the sample was determined at 535 nm and the TBARS concentration was calculated using the extinction coefficient, $1.56 \times 10^5\ M^{-1}\ cm^{-1}$.

Assay of glutathione (GSH)

GSH was measured in erythrocyte lysate. Erythrocytes were separated and washed three times in 5 ml of sterile 9 g/l NaCl solution and hemolyzed by diluting four-fold with water. Erythrocyte GSH concentration was determined according to the method of Beutler et al. (1963) using metaphosphoric acid for protein precipitation and 5,5′dithiobis 2-nitrobenzoic acid for color development. GSH concentration was calculated using $1.36 \times 10^4\ M^{-1}\ cm^{-1}$ as the molar absorption coefficient and was expressed as mg/g Hb.

Assay of total antioxidant capacity (TAOC)

TAOC was measured using a Randox 2332 kit, according to the method of Miller et al. (1993). ABTS (2,2′-azino-di-[3-ethylbenzthiazoline sulphonate]) is incubated with metmyoglobin and H_2O_2 to produce a radical cation with an absorbance at 734 nm. This absorbance is inversely proportional to the total antioxidant capacity of plasma.

Statistical analysis

All results were expressed as a median (range). Data were analyzed by non-parametric Kruskal–Wallis and Mann–Whitney U test. Correlations were analyzed by Pearsons test. The limit of statistical significance was set at $P < 0.05$.

Results

Biochemical data of the study groups at the end of the experimental period of 60 days and the statistical evaluation are shown in Table 1. $CuCl_2$ and STZ,$CuCl_2$ treated groups displayed significantly higher median serum Cu levels (227 and 222 μg/dl, respectively) when compared with the control group (189.1 μg/dl) ($P < 0.01$, $P < 0.01$, respectively). Although no significant difference was noted in median Cu levels between STZ,$CuCl_2$ treated (222 μg/dl) and STZ groups (212 μg/dl), a slight tendency to higher levels was apparent in the STZ,$CuCl_2$ treated group.

$CuCl_2$ treated group had significantly lower plasma glucose but higher GHb, TAOC and TBARS levels than the control group (147 vs. 169.5 mg/dl, $P < 0.05$; 4.95 vs. 3.07%, $P < 0.001$; 1.06 vs. 0.76 mmol/l, $P < 0.05$ and 9.23 vs. 6.85 μmol/l, $P < 0.001$, respectively).

STZ administered rats had significantly higher concentrations of both glucose and the early glycation product GHb than the control group (205 vs. 169.5 mg/dl, $P < 0.01$; 4.65 vs. 3.07%, $P < 0.001$, respectively). These findings reflected the establishment of diabetes mellitus in STZ administered rats. In

diabetic rats circulatory AGE-peptides were at significantly higher concentration than nondiabetic rats (21.9 vs. 14.1%, $P < 0.01$).

STZ,$CuCl_2$ group had significantly higher GHb, TAOC and TBARS levels compared with the control group (6.3 vs. 3.07%, $P < 0.001$; 1.1 vs. 0.76 mmol/l, $P < 0.05$ and 7.8 vs. 6.85 μmol/l, $P < 0.05$, respectively). Only plasma TAOC level was found to be significantly higher in the STZ,$CuCl_2$ treated group compared with the STZ group (1.1 vs. 0.87 mmol/l, $P < 0.01$). Pearson correlation analysis results are shown in Table 2.

In the $CuCl_2$ treated group, glucose was found to be negatively correlated with both GHb and AGEs ($r = -0.783$, $P < 0.05$; $r = -0.722$, $P < 0.05$, respectively); TBARS was observed to be positively correlated with AGEs ($r = 0.679$, $P < 0.05$). In the STZ group; copper was positively correlated with glucose and negatively with GSH ($r = 0.928$, $P < 0.05$; $r = -0.956$, $P < 0.05$, respectively); TAOC was found to be negatively correlated with AGEs ($r = -0.765$, $P < 0.05$) (Figs. 1, 2). In the STZ,$CuCl_2$ treated group; positive correlations were observed between Cu and TAOC ($r = 0.885$, $P < 0.05$) and between GHb and AGEs ($r = 0.914$, $P < 0.05$) (Figs. 3, 4, respectively), and negative correlations of GSH and with both glucose and TBARS ($r = -0.749$, $P < 0.05$; $r = -0.863$, $P < 0.05$).

Discussion

In this experimental study we investigated the effects of $CuCl_2$ intake on glycemic parameters, circulatory

Table 1 Biochemical parameters in the experimental groups median (minimum–maximum)

	Control	STZ	$CuCl_2$	STZ,$CuCl_2$
Cu (μg/dl)	189.1 (169.3–211.8)	212 (161.9–262.8)	227[a]** (212.4–269.8)	222[a]** (212.5–274)
Glucose (mg/dl)	169.5 (147–190)	205[a]** (190–276)	147[a]* (131–186)	195 (128–212)
GHb (%)	3.07 (2.12–4.23)	4.65[a]* (2.95–11.86)	4.95[a]*** (3.5–6.3)	6.3[a]*** (4.7–8.6)
AGE (%)	14.1 (11.12–19.21)	21.9[a]** (15.17–24.8)	17.7 (9.17–31.7)	13 (9.64–38.01)
TAOC (mmol/l)	0.76 (0.43–1.3)	0.87 (0.58–1.13)	1.06[a]* (0.79–1.34)	1.1[a]*,[b]** (0.99–1.52)
GSH (mg/gHb)	3.15 (2.5–4.41)	2.81 (1.32–3.87)	2.13 (1.26–4.44)	2.56 (1.37–5.75)
TBARS (μmol/l)	6.85 (5.67–8.28)	8.13 (5.6–10.8)	9.23[a]*** (7.5–13.1)	7.8[a]* (6.2–9.6)

[a] Versus control group

[b] Versus STZ group

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$

Table 2 Pearson correlation coefficients in $CuCl_2$ and STZ,$CuCl_2$ treated, STZ groups

Groups	Glucose	TBARS	Cu	TAOC	GHb
$CuCl_2$					
Ghb	$r = -0.783$*	–	–	–	–
AGE	$r = -0.722$*	$r = 0.679$*	–	–	–
STZ					
Cu	$r = 0.928$*	–	–	–	–
GSH	–	–	$r = -0.956$*	–	–
AGE	–	–	–	$r = -0.765$*	–
STZ,$CuCl_2$					
TAOC	–	–	$r = -0.885$*	–	–
GSH	$r = -0.749$*	$r = -0.863$*	–	–	–
AGE	–	–	–	–	$r = 0.914$*

* Correlation is significant at the $P < 0.05$ level

AGE-peptides and oxidative stress related parameters in diabetic and nondiabetic states. Previously, the beneficial effect of supplementation with $CuSO_4$ (20 μg/0.2 ml) intraperitoneally on every alternate day for 30 days) was reported in STZ-diabetic mice (Sitasawad et al. 2001). In this study, the rats were supplemented with $CuSO_4$ before multiple-dose STZ treatment (IDDM) and the effects were evaluated at 10 weeks. $CuSO_4$ supplementation prior to diabetes induction kept the levels of lipid peroxidation and blood glucose within the normal range (Sitasawad et al. 2001). However in another study high Cu intake (60 mg/kg of Cu, by gastric tube, twice a week, for 30 days) in diabetic condition has been reported to cause renal toxicity, high urinary glucose output and reduction in blood glucose levels (Galhardi et al. 2004). In the presented study we started the $CuCl_2$ treatment (4 mg Cu/kg body weight, subcutaneously on every alternate day for 60 days) on the third day of diabetes induction and continued it throughout the experimentation period of 60 days. $CuCl_2$ treated groups displayed significantly higher serum Cu levels when compared with the control group and a slight tendency to higher levels was also apparent in the STZ,$CuCl_2$ treated group when compared with the STZ group. In the STZ group on 60th of STZ injection, in accordance with hyperglycemia, GHb

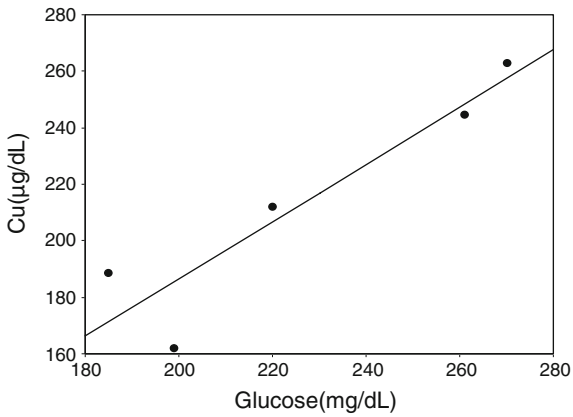

Fig. 1 Correlation between plasma copper and glucose in the "STZ" group (correlation coefficient $r = 0.928$; $P = 0.023$)

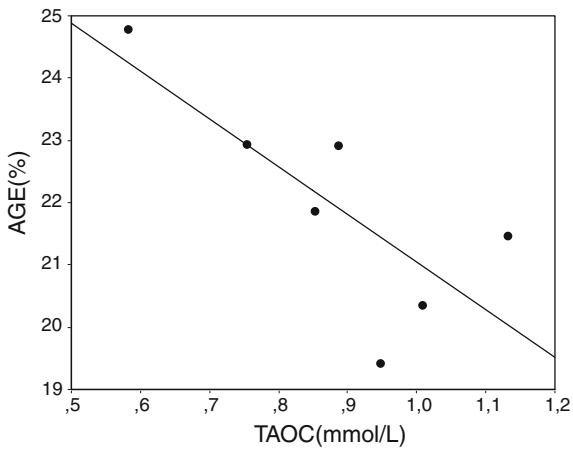

Fig. 2 Correlation between plasma TAOC and AGE in the "STZ" group (correlation coefficient $r = -0.765$; $P = 0.045$)

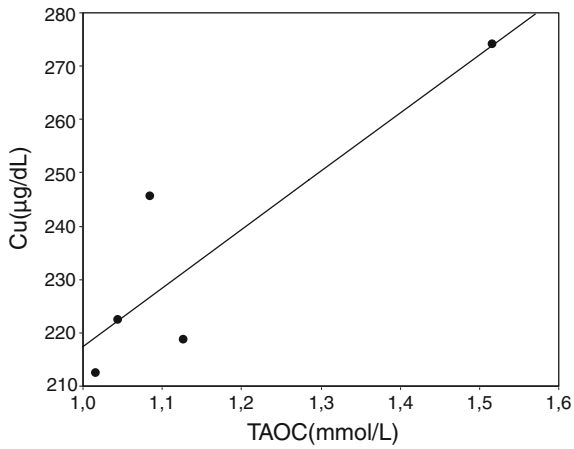

Fig. 3 Correlation between plasma copper and TAOC in the "STZ + $CuCl_2$" group (correlation coefficient $r = 0.885$; $P = 0.046$)

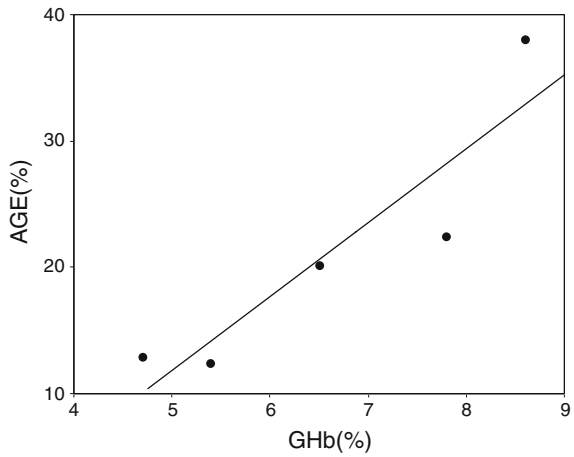

Fig. 4 Correlation between blood GHb and plasma AGE in the "STZ + $CuCl_2$" group (correlation coefficient $r = 0.914$; $P = 0.030$)

and circulatory AGEs were all at significantly higher concentrations compared with the control group. We noted a slight tendency to higher levels of both Cu and TBARS in the STZ group. Further, blood Cu was correlated positively with blood glucose and negatively with erythrocyte glutathione. The former correlation might be consistent with the increased release of Cu ions from the glycated form of Cu-containing enzymes in diabetic rats (Lin 1996) and the latter might reflect the increased GSH consumption by the increased free radical generation in the presence of Cu ions (Yoshida et al. 1995).

In the STZ,$CuCl_2$ treated group we observed that plasma glucose was negatively correlated with glutathione, which in turn was negatively correlated with TBARS. As stated above, high Cu intake in diabetic condition has been reported to reduce the blood glucose levels and this was explained by the increased urinary glucose output (Galhardi et al. 2004). The mechanism underlying renal toxicity was explained by oxidative stress and lipid peroxidation (Galhardi et al. 2004). We observed the highest GHb values and a positive correlation between GHb and AGEs in the STZ,$CuCl_2$ treated group. These findings are all consistent with the fact that hyperglycemia increases oxidative stress, lipid peroxidation and protein glycation (Abou-Seif and Youssef 2004).

As to the effects of $CuCl_2$ treatment in the nondiabetic state, we observed substantially lower plasma glucose but higher GHb levels in the treated rats, than the untreated. Furthermore within this group we observed that glucose was negatively correlated with both GHb and AGEs. These findings suggest the presence of factors other than glucose contributing to glycation of hemoglobin and the relative importance of this non-glucose contribution in $CuCl_2$ treated nondiabetic rats. We have noted a tendency to lower glucose but higher GHb values but in the STZ,$CuCl_2$ treated group when compared with the STZ group. In a very recently published paper, it had been shown that lipid peroxidation can contribute to glycation of hemoglobin in nondiabetic renal failure patients. It is well established that Cu ions induce lipid peroxidation (Selvaraj et al. 2005). Accordingly we observed that TBARS values were significantly higher in the $CuCl_2$ treated group than the control group. The highest TBARS and the lowest GSH values were recorded in this group. Additionally, TBARS was positively correlated with AGEs. Taken together, these findings are all indicative of adverse effects of $CuCl_2$ in the nondiabetic state. Metal-catalyzed oxidation reactions and glycation are closely related and potentially accelerate each other (Argirova and Ortwerth 2003).

On the other hand, we observed that total antioxidant capacity values was significantly higher in $CuCl_2$ treated groups than the untreated. Importantly, among all the parameters measured in this study total antioxidant capacity was the only parameter, displaying significant difference between STZ,$CuCl_2$ treated and STZ groups. The increase in total antioxidant capacity might be due to elevation in CuZnSOD activity. Trace elements such as Cu and Zn have been reported to be important in antioxidant defence (CuZn SOD) in diabetes mellitus (Eizirik et al. 1996; Sitasawad et al. 2001; Hussein et al. 2007).

To conclude, this experimental study provides evidence that copper intake increases total antioxidant capacity in both nondiabetic and diabetic states. However despite the potentiated antioxidant defence, lipid peroxidation and glycation enhancing effects of $CuCl_2$ are evident under nondiabetic conditions. Considering these effects, further studies addressing the effects of copper in diabetes are needed.

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