INDUCTION OF DIABETES MELLITUS ALTERS DISTRIBUTION OF SOME ESSENTIAL TRACE ELEMENTS AND ANTI-OXIDANT ENZYME STATUS IN FEMALE ADULT RATS

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ABSTRACT

To assess blood level and tissue distribution of essential trace elements such as copper, iron, molybdenum, selenium and zinc in experimentally induced diabetic and control rats after bolus injection of cocktail of essential trace elements and to assess the effect of diabetes induction on anti-oxidant enzyme status in female adult rats.Diabetes mellitus was induced in female adult Sprague Dawley rats by injection of streptozotocin. A cocktail containing twice physiological concentrations of Cu, Fe, Mo, Se and Zn was injected along with with antipyrine as internal reference marker were then injected intra- peritoneal to rats on 7th day after induction of diabetes. Blood samples of animals along with tissue samples from heart, liver, lungs, kidney and brain were then were collected after sacrificing the animals. Concentrations of various trace elements and antipyrine in various blood and tissue samples were determined. Activities of anti-oxidant enzymes SOD, GPx and total TAO were assessed.Concentrations of Cu, Fe, Mo, Se, Zn and antipyrine averaged 2808 ± 172 ug/L , 3725±662 ug/L , 14.20±1.20 ug/L, 64.20±3.90 ug/L, 595.2±52.2 ug/L and 161.5±7.2 mg/L in blood in control rats (n=8) while in the diabetic group (n=8) the values of corresponding trace element concentrations and antipyrine averaged 2701 +152 ug/L, 4592+525 ug/L, 18.8 +1.9 ug/L, 95.5+4.2 ug/L , 625+62 ug/L and 162.9+ 5.2 mg/L respectively. Unpaired student's t-test showed that Fe and Se levels were significantly higher (ANOVA Test; $p<0.05$) in the diabetic pregnant rats compared to controls. Distribution of Mo and Se were significantly higher in lungs and kidney of diabetic rates compared to control rats. Activties of TAO, GPX and TAO were significantly lower (Student/s t-test; $p<0.05$) in diabetic rats compared to controls.Although tissue distribution of selenium and molybdenum were significantly higher in some tissues of diabetic rats, considering the difficulty in extrapolation of results in diabetic rats to those in diabetic women, we urge exercising caution when comparing data from animal studies to human situations.

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INTRODUCTION

Diabetes Mellitus is well known to be associated with increased cardio-vascular, renal, neurologic and ocular complications (1, 2, and 3). In women diabetes mellitus is associated with maternal mortality and morbidity as well as with increased rate of congenital malformations (4, 5). We had reported earlier that disposition of some essential trace elements are altered in insulin-dependent diabetic mothers (6). Other investigators have also reported altered trace element concentrations in diabetic patients compared to control group (7). Although altered anti-oxidant status has been implicated in genesis of congenital malformations (8, 9) the effects of diabetes in distribution of essential trace elements in various tissues and on anti-oxdiant enzyme status have not been reported in detail humans or in animals to date. We had earlier explored the effect of induction of experimental diabetes in pregnant rats (10) on distribution of essential trace elements such as copper (Cu), iron (Fe), molybdenum (Mo), selenium (Se) and zinc (Zn) and compared the data with those obtained in control non-diabetic pregnant rats.

In this study we have investigated the effect of induction of diabetes mellitus in adult female rats under controlled experimental conditions, after bolus injection of these elements, seven days after induction of diabetes mellitus in diabetic and control animals to explore whether diabetes induction in female adult rats have similar or comparable effects on trace element distribution in blood and in various tissues and anti-oxidant enzyme status in adult pregnant rats (10). Besides our research group (10), this technique of experimental induction of diabetes mellitus in rats has been used by other research groups as well (11, 12).The essential trace elements Cu, Zn, Fe, Mo and Se were chosen for the study, considering their established role in anti-oxidant and protective functions in the body (13-15). Concentration of various trace elements in heart, lungs, liver, kidney and brain as well in blood of diabetic and control rats was assessed along with activities of antioxidant enzymes, SOD, GPX and TAO in blood samples of diabetic and control rats. Antipyrine was used as the reference internal marker considering its wide-spread use as internal reference marker in various studies (16, 17).

MATERIAL AND METHODS

Sprague Dawley rats (200-300 g body weight) raised in the animal house of the Faculty of Medicine, University of Kuwait were used for the study. Animals were divided into two groups of 8 rats each of study and control groups and fed with a standard laboratory diet and tap water was given ad libitum. They were fasted overnight prior to blood sugar determination and streptozotocin (STZ) injection, but were allowed free access to water during the duration of the study. On the first day of the experiment, diabetes mellitus was induced in study group rats by

single intraperitoneal injection of a buffered (0.1 M Citrate, pH 4.5) solution of STZ as described elsewhere (10,15) Control group of female rats were injected intraperitoneally with similar volume (100ul) of buffer as described by our research group earlier (10) . Induction of diabetes was assessed 48 hours after STZ injection, by determining glucose level in blood collected from tail tip of treated and control groups. Only those rats with a blood glucose level in excess of 200 mg/dl were recruited as the study group. On 7th day of induction of experimental diabetes, rats were anaesthetized with ether and were injected intraperitoneally with a bolus dose (150 ul) containing a cocktail of some essential trace elements (Cu concentration: 191.8 mg/L; Fe concentration: 81.4 mg/L; Mo concentration: 0.213 mg/L; Se concentration: 2.686. mg/L; Zn concentration: 26.28 mg/L; Total Bolus Volume: 150 ul) and antipyrine (concentration: 40 mg/L) used as reference marker. Control rats were injected with equal volume (150ul) of the above cocktail containing various trace elements and reference marker in concentrations mentioned above.

The injected rats were then grouped into 2 groups of 4 rats each. Two groups of 4 rats each belonging to control and study groups were then sacrificed by decapitation after 60 minutes respectively to permit equilibration of injected trace elements and reference marker, after trace element bolus injection. The blood samples were collected from cardiac puncture and tissue samples from liver, lungs, kidney, heart and brain were collected immediately after decapitation of animals, for analysis of various trace element and various biochemical parameters. Blood samples were collected from rats from heart by direct needle puncture, in special metal-free glass tubes, free of anticoagulant. Blood samples were allowed to clot and were centrifuged for 15 min at 2400 rpm. Serum was separated into metal- free plastic tubes and stored frozen at -20°C till analysis. The blood samples and tissue samples from control and study groups were pooled and stored separately at -20° C, till analysis.

Reagents

Only reagent grade chemicals (Sigma Chem. Co, USA) and were used for preparation of all solutions. Double—distilled water was used in all preparations. Standard solutions (1,000mg/L) of the elements were prepared by dissolution of pure metals or their salts (Merck, Darmstadt, Germany) and further diluted appropriately, prior to use.

Instrument

Atomic absorption spectrometer equipped with AA Analyst 300 graphite furnace (Perkin Elmer AA-914B, USA) was used for analysis of various trace elements in blood and tissue samples. Only HGA graphite tubes were used and signals were measured as peak areas and measurements calibrated using known trade element standards.

Analytical procedure

Following parameters were used for analysis of various trace elements: Cu (324.5nm wavelength and 0.7nm slit width), Fe (248.3nm wavelength, 0.2nm slit width) and Zn (2l3.9nm wave length, 0.7nm slit width). The serum samples were diluted (1:100) with deionized, double distilled water. Standards, prepared in deionized, double distilled water were run in the range of 30-240ppm, 10-40ppm, 5-20pmm for Cu, Fe and Zn respectively. Blood and tissue samples were diluted 5-fold by using deionised, double distilled water for Se (196nm wavelength, 2nm slit width) and Mo (313.3nm wavelength, 0.7nm slit width). The various trace elements standard solutions were prepared in deionized, double distilled water in the range of 10-40 ppm and 15-50 ppm for Mo and Se. The samples and standards were pipetted directly into the graphite tube. During analysis, internal argon flow rate through the graphite tuhc was kept at 250ml/min; gas flow was interrupted during atomization. Sample volume, ashing, atomization temperature, ramp and hold times were optimized before analysis to obtain maximum absorbance and minimum background. Palladium and magnesium nitrate were used as modifiers, in concentrations of 0.015 mg and 0.01 mg respectively. Most of the matrix was removed before the atomization step and less interference occurred during atomization. In order to validate the method for accuracy and precision, certified reference materials (quality serum control) were analyzed for each element. Repeatability and reproductivity tests were done routinely, with known reference trace element standards, to determine the validity and accuracy of our analytical technique. Glucose concentration of various samples was assessed using a glucometer ACCUTREND GC (Boerhinger Mannheim, Germany), Protein concentration in various serum samples was determined by Biorad protein assay (Biorad Labs, UK), measuring the absorbance at 595nm using UV-Visible spectrophotometer. Total cholesterol analysis was done using Infinity Cholesterol Reagent kit (Sigma Diagnostics, USA), measuring the absorbance wavelength between 500- 660nm. Concentration of triglycerides was assayed using Infinity Triglyceride Reagent Kit (Sigma Diagnostics), measuring the absorbance wavelength between 520-660 nm. Concentrations of antioxidant enzymes, namely superoxide dismutase (SOD), Glutathione peroxidase (GPx) and total anti-oxidant activit(TAO) in blood samples were determined spectrophotometrically (Randox Labs, UK).

Statistical analysis:

Statistical analysis of data was done using Stat-View 402 Statistics package, (SPSS 11 Windows, Microsoft, USA). Student's t-test or Mann-Whitney U-test Or ANOVA Test was used where appropriate. Results were considered significant with probability less than 0.05.

RESULTS

Parameters of control and diabetic group rats are given in Table I. Unpaired Student's t-test showed that blood sugar was significantly higher in diabetic group than control rats while levels of total protein cholesterol and triglycerides level of diabetic rats were not significantly different in diabetic group compared to control(Student's t-test ; $p>0.05$). No significant difference could be shown (Student's t-test; $p>0.05$) in body weight between the two groups as well.Cu, Zn, Fe, Se and Mo levels and reference marker concentration in blood of the control and diabetic groups of rats are shown in Table II. Concentrations of Cu, Fe, Mo, Se, Zn and antipyrine averaged 2808 ug/L, 3725 ug/L, l4.20 ug/L, 42.20 ug/L, 595.2 ug/L, and 161.50 mg/L in blood of control rats (n=8) while in the diabetic group, (n=8) the values of various trace element concentrations and antipyrine averaged 2701 ug/L, 4592 ug/L, 18.8 ug/L , 95.5 ug/L, 625 ug/L and 162.9 mg/L respectively. Unpaired student's t-test showed that Fe and Se levels were significantly higher ($p<0.05$) in the diabetic pregnant rats compared to controls. Cu, Mo and Zn values, however were not significantly different ($p>0.05$) between the two groups. Similarly concentration of reference marker, antipyrine was not significantly different ($p>0.5$) between control and diabetic groups.

Concentrations of various trace elements in heart, liver, lungs, kidney, brain and placenta of control and diabetic pregnant rats after 60 minutes of trace element bolus injection are shown in Table III and analysis was done using ANOVA test. Cu concentration in liver of diabetic group was significantly lower than that of control group ($p <0.05$) while the Zn concentrations in liver, heart and brain of diabetic group were significantly higher in diabetic rats compared to control rats ($p<0.05$). Cu and Zn concentrations of other tissues studied did not show any significant difference ($p>0.05$). Mo concentration in liver, lungs, kidney, brain and brain were significantly higher ($p<0.05$) in diabetic rats compared to control rats. Similarly Se concentration was significantly higher ($p<0.05$) in heart, lungs, kidney, heart and brain of diabetic rats compared to control rats as well. Iron concentration was significantly lower in liver, lungs, heart and kidney of diabetic rats ($p<0.05$), but the higher concentration of iron in brain of diabetic rats was not statistically significant ($p>0.05$).

Cu: Zn ratio in blood of control rats averaged 4.62±0.52 while that of diabetic group averaged 4.21±0.62. The lower in Cu: Zn ratio of diabetic group however was not statistically significant (Student's t-test; $p>0.05$).Cu: Fe ratio of control and diabetic groups averaged 0.72±0.25 and 0.56± 0.21 respectively. The lower Cu: Fe ratio of diabetic rats was not statistically significant as well (Student's t-test; $p>0.05$).

SOD activity in blood of control and diabetic rats are shown in Figure 1. Students; t-test showed that SOD levels of diabetic rats were significantly lower ($p<0.05$) than that of control group. GPx concentration in blood of control and diabetic rats are shown in Figure 2.

Students; t-test showed that GPx levels of diabetic group rats were significantly lower ($p<0.05$) than that of control group. Similarly the TAO activity in blood of diabetic rats were significantly lower than that of control group as well (Fig.3)

Table 1: Metabolic parameters of control and diabetic Rats.

	BLOOD SUGAR (mg/dl)	TOTAL PROTEIN (mg/dl)	TOTAL CHOLESTEROL (mmol/L)	TRIGLYCERIDES (mmol/L)	BODY WEIGHT (g)
CONTROL (n=8)	85.9±2.4	59.7±4.2	3.30±0.09	1.12±0.07	252±12
DIABETES (n=8)	332.3±36*	62.2±3.8	3.36±0±0.12	1.21±0.08	242±14

Statistical significance was assessed using student`s t-test.*$p<0.005$

Table 2: Concentrations of various trace elements and antipyrine in blood of control and diabetic Rats

TRACE ELEMENTS	CONTROL (n=8)	DIABETIC(n=8)
COPPER (µg/L)	2808±172	2701±152
IRON (µg/L)	3725 ±662	4592±525*
MOLYBDENUM(µg/L)	14.2±1.2	18.8±1.9
SELENIUM(µg/L)	64.2±13.9	95.5±4.2*
ZINC(µg/L)	595.2±52.2	625±62
ANTIPYRINE(µg/L)	161.5±7.2	162.9±5.2

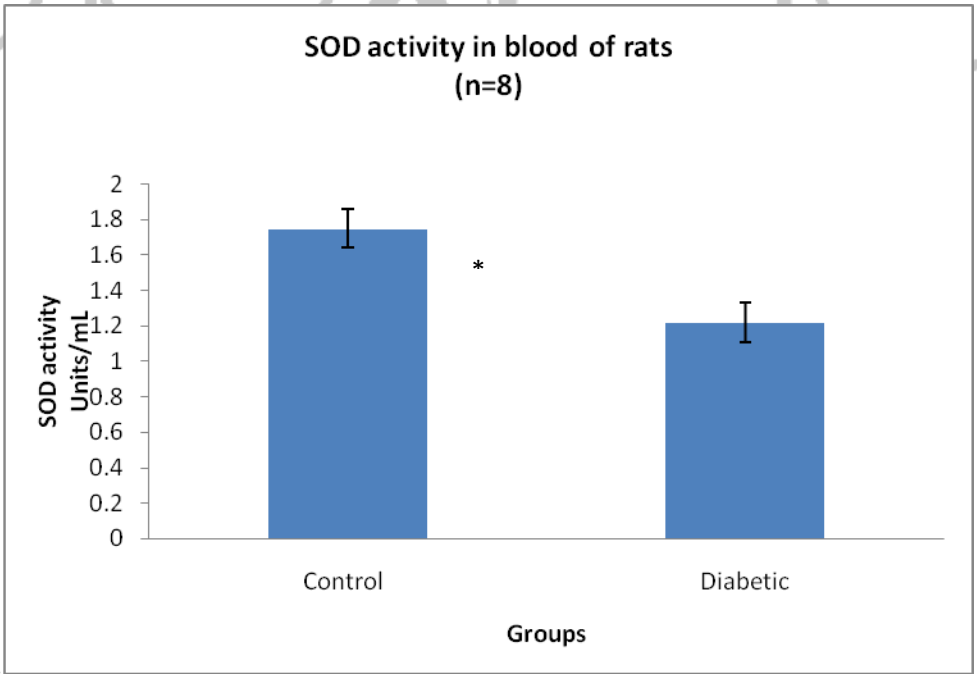
*$p<0.05$,Statistical significance was assessed by students t-test.

Table 3: Concentration of trace elements and antipyrine in various tissues of diabetic and control rats.

	TISSUE (µg/g)	COPPER (µg/g)	IRON (µg/g)	MOLYBEDENUM (µg/g)	SELENIUM (µg/g)	ZINC (µg/g)	ANTIPYRINE (mg/g)
CONTROL GROUP (n=8)	Heart	462± 75	1962±323	6.2±0.80	3.3±1.20	808±151	275±5.1
	Lung	363±42	1395±272	4.4±0.30	2.7±0.40	362±52	303±9.9
	Liver	445±141	2396±325	4.8±0.52	4.2±1.20	698±141	315±8.8
	Kidney	382±62	1475±202	4.2±0.30	2.82±0.35	705±101	292±8.9
	Brain	292±99	1402± 152	3.8±0.60	2.95±0.62	442±58	299±9.2
DIABETIC GROUP (n=8)	Heart	421±92	1192±525*	9.9±1.10*	7.1±0.50*	1352±162*	285±7.2
	Lung	242±52	998±363*	9.1±1.20*	9.9±1.60*	436±72	297±8.2
	Liver	232±99*	1792±299*	12.6±1.20*	4.32±1.40*	1499±225*	305±12.2
	Kidney	424±82	872±191*	11.9±0.80*	12.2±0.60*	692±112	303±11.8
	Brain	502±101	1799±162*	10.9±0.90*	7.9±1.60*	1395±292*	295±12.5

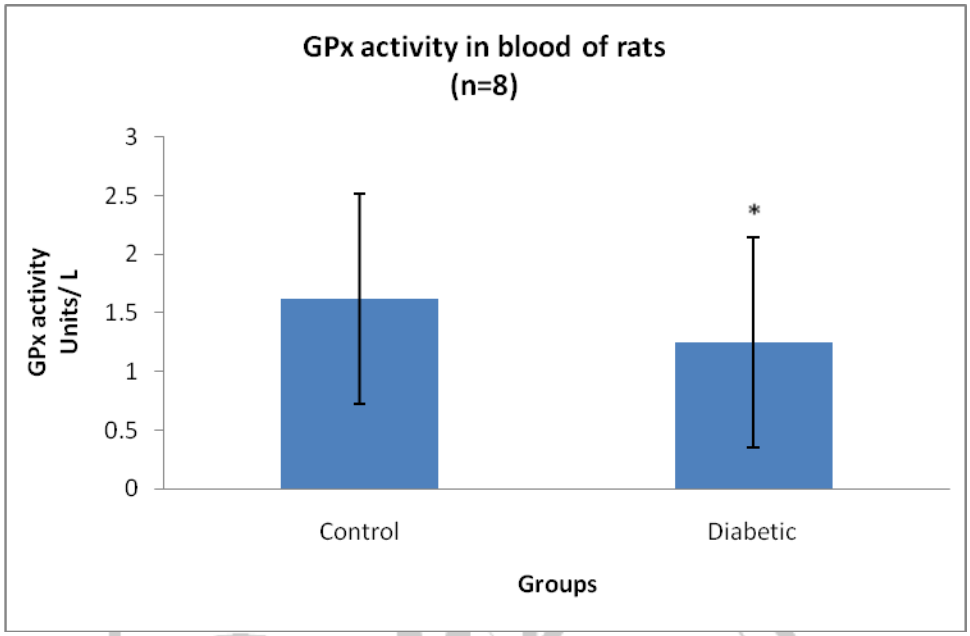
Statistical significance was assessed by Student's t-test. *p<0.05.

Figure 1: SOD activity in blood of control and diabetic rats.



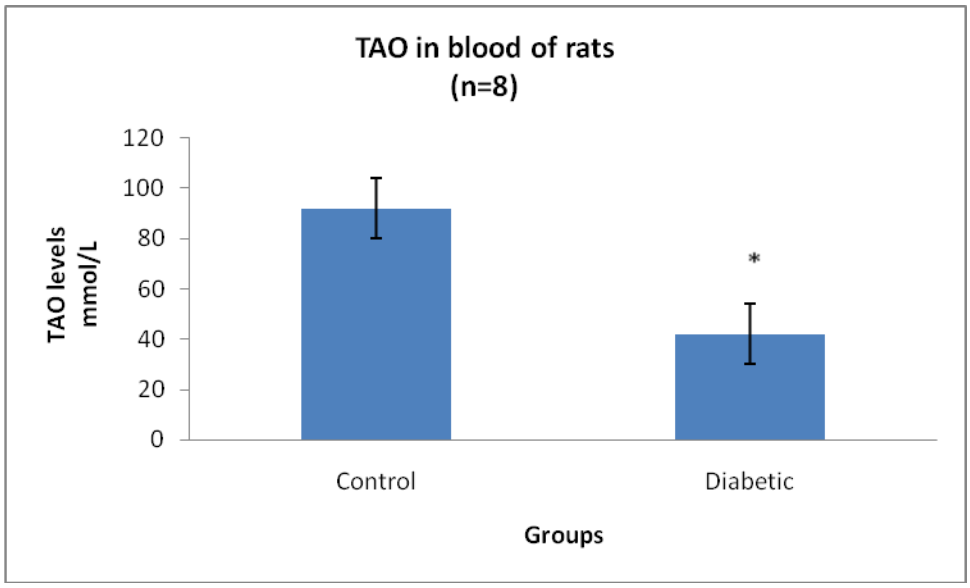
Statistical significance was assessed by Student's paired t-test. *P value<0.05. SOD: superoxide dismutase.

Figure 2: GPX activity in blood of control and diabetic rats.



Statistical significance was assessed by Student's paired t-test. *P value<0.05. GPx: glutathione peroxidase.

Figure 3: TAO activity in blood of control and diabetic rats.



Statistical significance was assessed by student's paired t-test. *P value<0.05. TAO: Total anti-oxidant status

DISCUSSION

The experimentally induced diabetic rat model has been used by several international research groups including our research group (10) as a valid animal model to study diabetes and we report for the first time data relating to disposition of some essential trace elements in various tissues in this model in female rats. Although data from animal studies are not directly comparable to human situations, an attempt has been made to explore whether areas of convergence or divergence can be identified between data from this animal model and those reported by some research groups in human diabetic patients.

No attempt has been made to find reference values for essential trace elements in the rat diabetic model and the study has focused more on the disposition of essential trace elements after injection of a fixed bolus dose of trace elements and reference marker. Since the control group of rats was subjected to the same mode of treatment like the diabetic group, it was premised that any statistically significant change in disposition or exchange characteristics of trace elements compared to reference marker can be attributed to the induced altered diabetic state of the animals compared to the untreated group.

Experimental induction of diabetes was found to alter the disposition of essential trace elements studied differently, in blood of diabetic rats compared to control rats. Thus Fe, Mo and Se level in blood of diabetic animals was significantly higher than that of control group rats while Cu and Zn levels were not significantly higher in study group than control group. We are unable to speculate whether on a larger population studied the higher Cu and Zn levels in diabetic group would have attained significance. But Cu level was significantly lower in liver of diabetic animals while selenium level was higher in all the tissues studied of diabetic group, after 60 minutes of bolus trace element load injection.

Previous studies from our research group relating to pregnant insulin-dependent diabetic women, however showed the copper level in blood to be higher in the diseased group than that of controls while no significant difference could be shown between diabetic and control women in the case of selenium. The higher selenium levels observed in diabetic rats contrasts with comparable non-significant trace element level observed by us in control and diabetic women at term. It is plausible that pregnancy situation has altered the trace element status differently compared to non-pregnant state, besides the problem of species difference impacting trace element distribution status differently. The higher iron level in diabetic rats however is in accord with a similar observation in insulin-dependent diabetic pregnant women. It is tempting to speculate that the rat diabetic model may be appropriate for study of Fe, Se, Zn and Mo maternal-fetal exchange and disposition and data comparable to human situations but inappropriate for

study of copper metabolism and disposition (10). The higher copper and lower zinc levels have been reported in a mixed population of male and female patients with insulin dependent diabetes as well (18). Other investigators (19) have also reported lower zinc levels in diabetic patients have been reported by other investigators as well. Higher copper level In diabetes has also been reported by Mateo et al and Walter et al W in this disease state in a pooled population of male and female patients, though in our study copper level in blood of diabetic rats was lower , though non-significantly than that of control rats (20, 21).

Cu: Zn ratio was lower in blood of diabetic rats compared to controls though the difference was not statistically significant. This contrasts with the findings of significantly higher Cu: Zn ratio observed by us in maternal blood of pregnant diabetic rats (10). However the Cu: Fe ratio observed by us in this study is comparable to the Cu: Fe ratio reported by us in the pregnant diabetic rats (10). Mo level in blood of diabetic rats were significantly higher than that of control group and this result was reflected in the higher Mo content in various tissues of diabetic rat as well. We are unable to speculate on the possible reasons for such a finding and to assess whether the higher molybdenum level in maternal and umbilical samples is a result of its reduced tissue utilization or altered maternal-fetal disposition or whether it is a reflection of modified anti-oxidant function in the diabetic state.

It is pertinent to note that Mo is a vital part of three important enzymes systems, namely xanthine oxidase, aldehyde oxidase and sulfatase and the higher molybdenum level in various tissues of diabetic rats compared to controls is compatible with the lower anti-oxidant enzyme activities of SOD, GPx and TAO in diabetic rats observed in this study. Oxidase and these enzyme systems have been proven to play crucial role in diverse functions as carbohydrate metabolism, uric acid and iron utilization, detoxification as well as antioxidant functions. Zinc levels were lower in the diabetic group compared to controls; however the difference was not statistically significant. It is worthy of note that high copper and low zinc levels have been reported in a mixed population of male and female patients with insulin dependent diabetes ‘H8. Lower zinc levels in diabetic patients have been reported by other investigators as well (I9). Interestingly, high copper level reported by us in pregnant diabetic patients is consistent with observations of other investigators 1910 in this disease state in a pooled population of male and female patients. This fact, combined with the significantly higher copper: zinc ratio observed by us in maternal blood of diabetic women are indicative of altered antioxidant mineral status of the diabetic group. Copper and molybdenum were significantly elevated in the umbilical vein of diabetic group as well. Induction of diabetes mellitus was shown to decrease the anti-oxidant enzyme activities as well as total anti-oxidant status in female rats and this finding was in accord with the

altered anti-oxidant function in pregnant diabetic rats as well (10).

We conclude that levels of some essential trace elements and antioxidant mineral function are altered in the experimentally-induced diabetic rats. Further, our results seem to indicate that the rat diabetic model may not be the appropriate animal model to study the role of nutritional factors, more specifically the role trace elements and antioxidants in this disease situation since the results are at variance with many findings reported in human disease states. However, on issues of convergence the model may be considered suitable for the study. Unpublished data from our laboratory seem to indicate that there are significant alterations in levels of antioxidant enzymes, superoxide dismutase, glutathione peroxidase and total antioxidant activity in blood and placentae of diabetic pregnant women as well. In the light of higher incidence of congenital malformations reported in infants of diabetic mothers, greater attention needs to be given for adequate supply of essential trace elements in diet of diabetic pregnant women, to ward off possible ill effects for the fetus as well as the newborn. Further studies in this direction in experimental animals as well as in human diabetic states are in progress.

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