

**COBALT AND ASCORBIC ACID TREATMENT: EFFECTS ON
TISSUE NITRIC OXIDE LEVELS IN STREPTOZOTOCIN-
DIABETIC RAT**

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ABSTRACT

The aim of present study was to determine the effect of cobalt (II) chloride (CoCl_2) and CoCl_2 plus ascorbic acid (AA) on liver and kidney nitric oxide level at different stage of diabetes. Three days after streptozotocin (STZ) injection, rats were divided into six groups: control, diabetic, control and diabetic treated with cobalt chloride, control and diabetics treated with cobalt chloride plus ascorbic acid. 0.5 mM CoCl_2 or 0.5 mM CoCl_2 plus 1 g/l ascorbic acid were added to their drinking water daily. At the end of the second, fourth and sixth week of the treatment all animals were sacrificed by decapitation. Liver and kidney tissues were removed for the determination of nitric oxide (NO) levels. Cobalt treatment effectively decreased the nitric oxide levels both in liver and kidney tissues of diabetic rats. Thus, the effect of oral administration of cobalt treatment may prove effective in improving the impaired nitrite status during the early stage of diabetes and ascorbic acid supplementation at this dose (1g/l) potentiates the effectiveness of cobalt action.

KEYWORDS: Nitric oxide; diabetes; cobalt; ascorbic acid; liver and kidney

INTRODUCTION

Diabetes affects a variety of metabolic processes and characterized by alterations in tissue trace element and antioxidant concentrations which causes oxidative damages. Hyperglycemia is associated with excessive free radical generation and oxidant stress and reduction in the antioxidant status.

In recent studies, *in vitro* and *in vivo* insulinlike activity of several elements were showed by researchers (Abrams and Murrer, 1993; Brichard and Henquin, 1995; Novelli and Rodrigues, 1988; Özçelikay et al., 1996; Shisheva, Gefel and Shechter, 1992). Saker and co-workers (1998) were determined the potential role of enhanced tissue glucose uptake vs. decreased systemic glucose production in mediating the glycemia-lowering effect of cobalt chloride (CoCl_2) in diabetic rats. In principle, the glycemia-lowering effect of CoCl_2 could be decreased systemic glucose production, increased tissue glucose uptake, or a combination of the two mechanism. It has been previously reported that addition of 2 mM Co(II) in drinking water reduced the glycemia of streptozotocin-induced diabetic rats (Ybarra, Behrooz, Gabriel, Köseoğlu and Ismail-Beigi, 1997). Its action has been mainly ascribed to increased GLUT1 expression (Ybarra et al., 1997) and inhibition of gluconeogenesis (Saker et al., 1998) in STZ-diabetic rats. On the other hand, hydrophilic vitamin, ascorbic acid is used as a scavenger of reactive oxygen species. In aqueous solutions, ascorbate also scavenges reactive nitrogen oxide species efficiently. A sufficient supply of antioxidants either from the diet or from supplements might help to prevent or delay the occurrence of oxidative burden (Behrens and Madere, 1991; Siman and Eriksson, 1997). Cobalt alone or with a combination of ascorbate has been previously demonstrated to decrease lipid peroxidation in STZ-diabetic rats in various organs such as the liver, kidney (Yıldırım and Büyükbingöl, 2002, 2003a), heart and aorta (Yıldırım and Büyükbingöl, 2003b).

NO is a free radical gas that is synthesized from L-arginine by three different isoforms of NO synthase (NOS). NO plays an important role in homeostatic vasodilatation and regulation of blood flow, but excess release induces tissue disorders because of increased oxidative stress, especially caused by the production of peroxynitrite. It has been reported that NO is responsible for various vascular complications such as coronary arteriosclerosis and diabetes (Ignarro, Buga, Wood, Byrns and Chaudhuri, 1987; Ischiropoulos, Zhu and Beckman, 1992; Moncada and Higgs, 1993; Satoh, Fujimoto, Haruna, Arakawa, Horike, Komai, Sasaki, Tsujioka, Makino and Kashihara, 2005). NO is extremely unstable and easily oxidized, it is difficult to measure NO levels *in vivo*. Recently, evaluations of the stable NO end products nitrite (NO_2^-) and nitrate (NO_3^-), collectively

NO_x, in biologic fluid, based on the Griess method, have been widely conducted to estimate NO production.

The objective of this study was to determine the effects of cobalt chloride and cobalt chloride plus ascorbate on nitric oxide levels which accompanies with diabetes. The levels of nitric oxide were measured in liver and kidney tissues of both control and diabetic rats (2 to 6 weeks).

MATERIALS AND METHODS

Animals

Diabetes was induced in male Sprague Dawley rats weighing 200-220 g, by a single intraperitoneal injection of streptozotocin (STZ, 45 mg/kg) prepared in 0.1M citrate buffer (pH 4.5). Control animals were injected with the buffer (0.1 ml/100 g) only. Diabetic state was confirmed by detecting the presence of hyperglycemia in blood drops obtained following tail snipping at 48 and 72 h after STZ injection, using Reflolux (Boehringer Mannheim, East Sussex, UK) glucometer and test strips. The minimal blood glucose value accepted for a diabetic rat was 13.9 mM. 2, 4 and 6 weeks of diabetes were studied. All animals were housed in air/humidity-controlled room, with 12 h dark/light cycle, and allowed food and water (control animals only) *ad libitum* throughout the experiment.

Animals were randomly assigned to 6 groups for each week: (1) C, control group (n=7), (2) D, diabetic group (n=9), (3) CCo, control group with cobalt treatment (n=7), (4) DCo, diabetic group with cobalt treatment (n=9), (5) CCoAA, control group with cobalt plus ascorbic acid treatment (n=7) and (6) DCoAA, diabetic group with cobalt plus ascorbic acid treatment (n=9). 0.5 mM cobalt (II) chloride were added to the drinking water of the CCo group, on the other hand, 0.5 mM cobalt (II) chloride and 1 g/l ascorbic acid were added to the CCoAA and DCoAA's drinking water every day.

Tissue Sample Preparation

2, 4 and 6 weeks after STZ injection, animals were sacrificed by decapitation without anaesthesia. Liver and kidney tissues of each rat were immediately removed, cleaned of gross adventitial tissue, blotted dry and placed in aluminum foil and stored at -80°C and assayed within 3 weeks. Tissue

samples were homogenized in 3 volumes of % 1.15 KCl at 4°C for 30 sec (2x15 sec with 1 min cooling interval) at 3000 x g using a Teflon glass pestle with Heidolph homogenizer and centrifuged at 20,000 x g for 15 min. and supernatant was kept at -80°C for determination of NO levels.

Experimental Procedure

Nitric oxide, as an unstable molecule, that reacts with oxygen and biological molecules to form several end products (e.g. nitrite, nitrate and S-nitrosothiols) that can be determined by different methods (Braman and Hendrix, 1989; Mesaros, 1999). The most preferred method is based on Griess reaction which can be easily applied in the laboratory. The tissue sample homogenized as mentioned above (0.5 ml) was incubated with nitrate reductase (EC 1.6.6.2) from *Aspergillus* sp. (50 mU/100µl of sample) with NADPH (final concentration, 80 µmol/l) diluted in 20 mmol/l Tris buffer (pH 7.6) for 30 min at room temperature for nitrate reduction (Guevara, Iwanejko, Dembinska-Kiec, Pankiewicz, Wanat, Anna, Golabek, Bartus, Malczewska-Malec, and Szczudlik, 1998). After the reduction, 5% (w/v) ZnSO₄ was added for deproteinization. Then this mixture was centrifuged at 5000 x g for 10 min. The samples nitrite levels were measured by Griess reaction (Green, Wagner, Glogowski, Skipper, Wishnok and Tannenbaum, 1982). The absorbance at 540 nm was measured. Nitric oxide levels were calculated from the standard curve constructed using known nitrite concentrations. Tissue nitrite levels were expressed as µmol g⁻¹ wet weight.

All experiments were performed using at least 7 animals in each group and the results were expressed as mean ± standard deviation (SD). Data were analyzed using Duncan's multiple range test. A p value of < 0.05 was considered significant.

RESULTS

Induction of diabetes by STZ was confirmed by the presence of glucosuria within 24 hr. The general biochemical parameters and body weights in control and diabetic groups are shown in Table 1. As expected, diabetic animals were hyperglycemic (minimal blood glucose level was 13.9 mM) and their body weights were decreased, while nondiabetics were

normoglycemic and high body weight. Data also indicated that the plasma cholesterol and triglyceride levels were elevated in diabetics.

General features of the experimental model

		CONTROL	DIABETIC
2 WEEKS	Glucose (mM)	6.8 ± 0.5	23.5 ± 3.9*
	Cholesterol (mM)	1.7 ± 0.3	3.8 ± 0.4*
	Triglycerides (mM)	1.1 ± 0.2	8.4 ± 1.3*
	Body weight (g)	249 ± 6	220 ± 8*
	Fluid intake (ml)	44 ± 5	170 ± 10*
4 WEEKS	Glucose (mM)	7.3 ± 0.6	25.8 ± 5.9*
	Cholesterol (mM)	1.8 ± 0.5	4.3 ± 0.3*
	Triglycerides (mM)	1.2 ± 0.5	9.1 ± 2.7*
	Body weight (g)	273 ± 7	212 ± 5*
	Fluid intake (ml)	48 ± 4	160 ± 8*
6 WEEKS	Glucose (mM)	7.8 ± 0.6	27.3 ± 3.3*
	Cholesterol (mM)	1.7 ± 0.3	4.2 ± 0.7*
	Triglycerides (mM)	1.3 ± 0.1	8.9 ± 2.1*
	Body weight (g)	288 ± 12	232 ± 13*
	Fluid intake (ml)	52 ± 5	168 ± 10*

All values are means \pm SD. Each group consisted of eight animals. Significance of difference was assessed by Duncan's test. p values are shown as * $p < 0.05$ vs. control groups.

At the end of second week, nitrite levels were determined in liver and kidney tissues of all groups (Fig 1). In diabetic rat liver and kidney, we determined 254% and 293% increase in nitrite levels, respectively. Increased levels of nitrite were lowered 21% by CoCl_2 and CoCl_2 plus AA treatment.

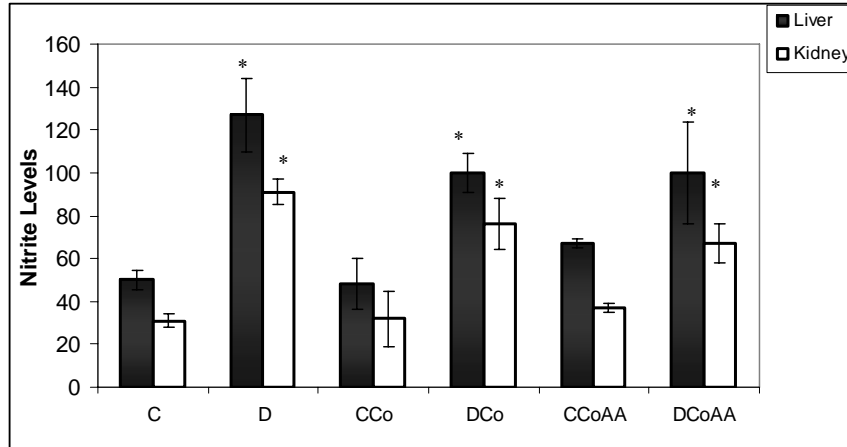


Figure 1. Nitrite levels ($\mu\text{mol g}^{-1}$ wet weight) of liver and kidney tissues *at the end of second week*. Control (C), diabetic (D), cobalt treated control (Co), cobalt treated diabetic (DCo), cobalt plus ascorbic acid treated control (CCoAA) and cobalt plus ascorbic acid treated diabetic (DCoAA) groups. (Results are expressed as means \pm SD (n=7 for C, Co and CoAA; n=9 for D, DCo and DCoAA groups). p values are shown as * $p < 0.05$ versus control groups. $p < 0.05$ versus diabetic groups)

At the end of 4th week we determined significant decrease ($p < 0.05$) in diabetic liver and kidney nitrite levels comparing to controls (Fig 2). In diabetic liver which is treated by CoCl_2 , we determined 47% decrease in

nitrite levels comparing to control group. CoCl_2 plus AA treatment also made a significant reduction ($p < 0.05$) in diabetic liver nitrite levels (56%).

We also demonstrated a significant decreases ($p < 0.05$) in diabetic kidney nitrite levels, with the treatment of CoCl_2 and CoCl_2 plus AA (Fig 2). 24% and 48% reduction were investigated respectively comparing with the control group.

At the end of 6th week we determined liver and kidney nitrite levels (\pm SD) in control, diabetic, treated controls and treated diabetic groups (Fig 3). The nitrite levels of the CoCl_2 and CoCl_2 plus AA treated diabetic rats exhibited a statistically significant recovery in both tissues. CoCl_2 with a combination of AA treated diabetic rats, exhibited 75% recovery for liver and 59% for kidney approaching to control levels. However, nitrite levels increased progressively in diabetic group and significantly higher as compared to controls at all studied times.

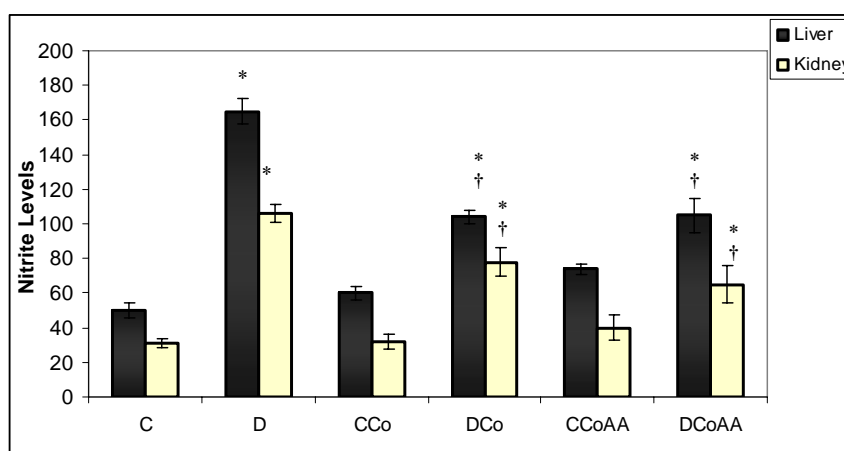


Figure 2. Nitrite levels ($\mu\text{mol g}^{-1}$ wet weight) of liver and kidney tissues *at the end of fourth week*. Control (C), diabetic (D), cobalt treated control (Co), cobalt treated diabetic (DCo), cobalt plus ascorbic acid treated control (CCoAA) and cobalt plus ascorbic acid treated diabetic (DCoAA) groups (Results are expressed as means \pm SD

(n=7 for C, Co and CoAA; n=9 for D, DCo and DCoAA groups). p values are shown as * p < 0.05 versus control groups. † p < 0.05 versus diabetic groups).

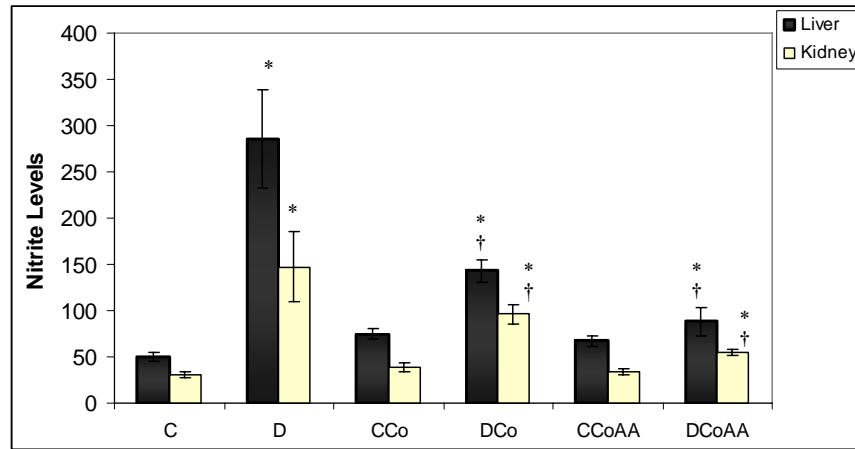


Figure 3. Nitrite levels ($\mu\text{mol g}^{-1}$ wet weight) of liver and kidney tissues *at the end of sixth week*. Control (C), diabetic (D), cobalt treated control (Co), cobalt treated diabetic (DCo), cobalt plus ascorbic acid treated control (CCoAA) and cobalt plus ascorbic acid treated diabetic (DCoAA) groups (Results are expressed as means \pm SD (n=7 for C, Co and CoAA; n=9 for D, DCo and DCoAA groups). p values are shown as * p < 0.05 versus control groups. † p < 0.05 versus diabetic groups).

DISCUSSION

The biological importance of nitric oxide has been reported by several investigators (Ignarro, et al., 1987; Ischiropoulos, Zhu and Beckman, 1992; Moncada and Higgs, 1993; Satoh, Fujimoto, Haruna, et al., 2005). Excess or deficient NO has been related to several pathological conditions. Corbett et al. indicated that, NO formation may play a role in the destruction of the pancreatic β -cells during the development of diabetes (Corbett, Mikhael, Shimizu, Frederick, Misco, McDaniel and Kanagawa, 1993). Under diabetic conditions, free radicals such as O_2^- and NO are produced as a result of the

induction of the glycation reaction in β -cells that have been affected by diabetic oxidative stress. NO interacts with O_2^- to form the highly reactive hydroxyl radical that leads to reactive oxidative damage (Beckman, Beckman, Chen, Marshall and Freeman, 1990). It is also well established that the increase in glucose blood levels in diabetes mellitus increase the cytosolic levels of NADPH oxidase resulting in an increase in nitric oxide production (Grisham, Jourd-Heuil and Wink, 1999).

In our recent study, we have demonstrated increased nitrite levels of diabetic and treated groups ($p < 0.05$) compared to control groups. In addition, NO targets intracellular antioxidative enzymes, resulting the loss of their function. At the end of 2nd, 4th and 6th weeks treatment with cobalt chloride and cobalt chloride plus ascorbic acid, nitrite–nitrate levels were significantly decreased compared to diabetic group ($p < 0.05$) (Fig. 1, 2 and 3). Our previous studies indicate that the free radical scavenging property of $CoCl_2$ and $CoCl_2$ plus AA suggests that $CoCl_2$ might protect against diabetic oxidative stress (10-12). Low molecular weight compounds potentially capable to remove excess pathogenic NO due to NO overproduction. It has been reported that NO binding is followed by oxidation of Co(II) to Co(III) with the formation of a Co-NO complex formally containing Co(III) (Rochelle, Morana, Kruszyna, Russell, Wilcox, Smith, 1995; Kruszyna, Megyar, Rochelle, Russell, Wilcox and Smith, 1998).

In present study, we have shown that there is a decrease in the level of nitric oxide by the treatment with cobalt alone and vitamin C plus cobalt in liver and kidney tissues during the progression of diabetes. Our study was done at different stages of diabetes to determine whether there is a significant difference in cobalt chloride and cobalt chloride plus ascorbic acid treatment on nitrite levels. The nitrite content in control group is remained unchanged throughout the 6th week diabetic group the nitrite levels were increased up to 6th week comparing with control groups. Kidney nitrite level was decreased at 2nd week because of advanced glycation end products which have oxidizing potential can quench nitric oxide as reported in Bucala and his colleagues research (Bucala, Tracey and Cerami, 1991). At 4th and 6th weeks renal nitrite level was increased because of some disturbances in amino acid metabolism in diabetes may contribute to increased intrarenal NO production. Increased glucose concentration in diabetes induces elevation in intracellular levels of diacylglycerol activates protein kinase C, which has been shown to activate nitric oxide synthases.

Studies on patients with diabetes mellitus, a disorder associated with hyperglycemia and accompanying complications, have revealed oxidative stress loads. Superoxide, hydrogen peroxide, hydroxyl radical and singlet oxygen which are named as reactive oxygen species have been implicated to play important roles in diabetes (Halliwell, 1997; Baynes and Thorne, 1999; Opara, 2002). NO may protect against reactive oxygen species from the tissue (Wink, Vodovotz, Grisham, Degraff, Cook, Pacelli, Krishna and Mitchell, 1999). It has been also indicated that, nitric oxide is an important mediator, governing a range of physiological functions in animals, from controlling smooth muscle tone in the cardiovascular, gastrointestinal, respiratory and genitourinary systems, to neurotransmission and a role in immune function and inflammation (Singh and Evans, 1997).

Diabetes mellitus is associated with increased lipid peroxidation, which may contribute to long-term tissue damage and increased oxidative stress (Yıldırım and Büyükbingöl, 2002; 2003a; 2003b). Our data have shown that administration of the cobalt and cobalt plus ascorbic acid to diabetic rats decreased the tissue levels of nitrite significantly, indicating that it may be useful for correcting oxidative stress.

ÖZET: Bu çalışmanın amacı kobalt II klorür (CoCl_2) ve kobalt II klorür ile birlikte verilen askorbik asitin (AA) diyabetin çeşitli haftalarında karaciğer ve böbrek nitrik oksit düzeylerine olan etkisini belirlemektir. Streptozotolin (STZ) uygulamasından üç gün sonra sıçanlar altı gruba ayrılmışlardır: kontrol, diyabetik, kontrol ve diyabetiklerin kobalt klorür ile tedavili grupları, kontrol ve diyabetiklerin kobalt klorür ve askorbik asit ile tedavili grupları. 0.5 mM CoCl_2 veya 0.5 mM CoCl_2 artı 1 g/l askorbik asit, sıçanların günlük içme suyuna katılarak verilmiştir. Çalışmanın ikinci, dördüncü ve altıncı haftaları sonunda sıçanlar anestezi altında öldürülmüşlerdir. Karaciğer ve böbrek dokuları nitrik oksit (NO) düzeyleri belirlenmek amacı ile çıkarılmıştır. Kobalt tedavisinin hem karaciğer hem de böbrek dokusunda nitrik oksit düzeylerini etkili bir şekilde azalttığı bulunmuştur. Bu durum, oral yolla kobalt alımının diyabetin erken döneminde bozulan nitrit düzeylerini normale döndürmede etkili olduğunu ve askorbik asit ilavesinin de belirtilen dozda (1g/l) kobalt etkisine katkıda bulunduğunu göstermektedir.

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