Ameliorating effect of cobalt chloride on renal failure and glucose lowering effect in diabetic nephropathy induced in uninephrectomized diabetic rat

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Abstract

Diabetic nephropathy, a progressive development of renal insufficiency in the setting of hyperglycemia, is the major single cause of chronic renal failure (CRF) in which hypoxia plays a critical role. This study evaluated the efficacy of cobalt chloride, a Prolyl 4-hydroxylase (PHD) inhibitor, in amelioration of renal injury, as well as its effect on hyperglycemia in uninephrectomized diabetic rats. The effect of cobalt chloride (CoCl2, 10 mg/kg, i.p. OD) treatment on plasma urea, creatinine, uric acid, electrolytes like sodium, potassium, chloride, as well as blood glucose levels were checked along with measurement of the dry weight of contralateral kidney in different groups. A significant rise in plasma urea, creatinine and uric acid levels was observed in uninephrectomized diabetic rats. Cobalt chloride (10 mg/kg, i.p. OD) treatment for seven continuous days, followed by intermittent dosing for 30 days, showed improvement in renal injury by a significant fall in the plasma urea, creatinine and uric acid levels with restoration to partially normal values as compared to an uninephrectomized uninephrectomized diabetic group. A significant change in plasma electrolyte levels was observed which was partially normalized in the cobalt chloride group along with a reduction in the dry weight of kidney. A significant decrease in the blood glucose level was observed in the CoCl2 treated group as compared to the uninephrectomized diabetic group. Our study shows the effect of CoCl2 in amelioration of renal failure and anti-hyperglycemic effect.

Introduction

Diabetic nephropathy is the most recurrent cause of end-stage renal disease (ESRD) in developed countries.1 Hypoxia plays a critical role in the pathogenesis and progression of chronic renal failure.2 Regulatory mechanisms are exerted by hypoxia by influencing gene expression through a family of transcription factors known as hypoxia inducible factors (HIFs).3 HIFs are heterodimers composed of two different oxygen-dependent α-subunits and a constitutive β-subunit, regulation of which is exerted by oxygen-dependent prolyl hydroxylation of the α-subunit. HIFs govern transcriptional activity of a host of genes which are cell/tissue protective.4-7 Irrespective of the underlying cause, there is growing evidence to suggest involvement of regional renal hypoxia in the pathophysiology of acute kidney injury (AKI).8,9 Furthermore, chronic hypoxia appears to play an important role in the progression of chronic renal failure.10 Diabetes is certainly a chief risk factor and a leading cause of end-stage kidney disease in developed countries.11

Compelling evidence points to induction of an early hypoxia in diabetic kidneys. A study utilizing blood oxygen level dependent (BOLD)-MRI has shown that kidneys of streptozotocin-induced diabetic rats are hypoxic even at an early stage.12 In a hypertensive type 2 diabetic nephropathy rat model (SHR/NDmc-r-cp), it has been documented that the accumulation of pimonidazole, a compound incorporated into hypoxic cells leads, to renal hypoxia.13 Furthermore, a study of intrarenal haemodynamics in human type 2 diabetic patients showed correlation between a decreased peritubular capillary flow and tubular dysfunction, thus supporting pathogenic role of chronic hypoxia in diabetic kidney.14 Under hypoxic conditions, HIF instead of being hydroxylated, transactivates in the nucleus a host of genes involved in the adaptation to hypoxia.15 Interestingly, cobalt inhibits HIF degradation by PHDs, thus enhancing HIF activity.16

This study was designed to explore the role of cobalt chloride induced augmentation on HIF activity. While our ultimate goal was to treat chronic hypoxia in diabetic nephropathy induced chronic renal failure, in this study we used a model of unilateral nephrectomy followed by STZ treatment to investigate the effects of our approach in ameliorating renal failure. We tested a hypothesis that administration of cobalt chloride retards the progression of renal failure and improves renal function with a significant decrement in the plasma glucose levels to which we obtained consistent results.

Materials and Methods

Animals

Eight-week old Sprague-Dawley (SD) rats from Torrent Research Centre, Bhat, Gujarat, India, were maintained in well controlled supplied air, humidity (<70%) and temperature (<30˚C) with a 12 h day and night cycle at the Central Animal Facility, Nootan Pharmacy College, Visnagar, India (CPCSEA n. 1244/ac/08/CPCSEA). Each rat was individually housed in a plastic box cage and had free access to untreated tap water and standard rat chow (Pranav Agro Ltd., Ahmedabad, India) according to the norms of IAEC (Institutional Animal Ethics Committee) and CPCSEA. After surgery, animals were inspected daily for level of activity and healing of surgical wounds.

Induction of diabetic nephropathy by unilateral nephrectomy followed by STZ administration

All rats were initially subjected to removal of the right kidney to accelerate the development of diabetic nephropathy, as described previously.17 Male SD rats were anesthetized by intraperitoneal injection of a mixture of ketamine (60 mg/kg ip) and xyazine (6.5 mg/kg ip). The anesthetized rats were placed on their ventral surface on a homeothermic heating pad; a dorsovenal incision parallel to spinal cord was made in the skin and muscle layer. The right kidney was freed gently of connective tissue and pulled out by grasping the perirenal fat. A silk thread was passed from just above between the renal artery and ureter, and a knot was tightened, the right kidney was removed giving
a cut before the knots at the artery and ureter, respectively, and the cavity closed by double sutures of muscle and skin, allowing animals to recover. Post-operative care included administration of normal saline (2 mL/animal), application of povidone, buprenorphine HCI (0.03 mg/kg, s.c. o.d./3 dyas) and benzyl penicillin (20,000 IU/kg, i.m, bid/3 days).

One week post surgery, a single intraperitoneal injection of streptozotocin (Future Delhi, India) (40 mg/mL in 0.1 mol/L phosphate/0.4 mol/L citrate buffer; pH 6.5) at a dose of 45 mg/kg body weight was injected in the uninephrectomized animals. The animals had free access to water at all times and feed was available ad libitum. Diagnosis of diabetes was established 48 h after the streptozotocin injection by determination of the tail vein blood glucose concentration by using a glucometer. Any streptozotocin-treated animal which at this time had a 4-6 h fasting blood glucose concentration of less than 200 mg/dL was eliminated from the study.

Sham surgery
Animals in the Sham group underwent the same surgical procedure as above but the kidney was neither cut or removed, only fatty material was removed and the kidneys were touched with forceps and threads. Similar post-operative care procedures were followed. After surgery, rats were placed individually in cages with free access to food and water.

Grouping of animals
Animals were randomized according to body weight and divided into four groups as follows before surgery: i) control animals (n=06); ii) animals with sham surgery (n=06); iii) animals with unilateral nephrectomy followed by STZ administration (n=06) without treatment (uninephrectomized diabetic); iv) animals with unilateral nephrectomy followed by STZ administration (n=06) with CoCl2 treatment (treatment group).

Estimation of parameters
Biochemical parameters
Blood samples were collected at basal and after 1, 3 and 5 weeks of study from a sublingual vein. The plasma was separated by centrifugation at 4000 rpm for 10 min at 4°C and was used to estimate creatinine (Jaffé method), uric acid and urea (Kinetic UV test) by semi-autoanalyzer (Erba Mannheim’s) and methods described previously.

Plasma electrolytes
Plasma sodium (Na) and potassium (K) concentrations were determined by standard flame photometry and chloride (Cl) by the method of Schales and Schales.

Estimation of plasma glucose (mg/dL)
Blood was withdrawn from tail vein and glucose level was estimated at basal, after 48 h and at the fifth week by commercially available glucose kits (Horizon®, OneTouch – Johnson & Johnson, India) based on a glucose oxidase method.

Change in the dry contralateral kidney weight
Changes in dry weight of contralateral kidney were estimated by comparing the weight of the kidney with that from an animal which had undergone a sham nephrectomy. Change in the weight of CoCl2 treatment group was estimated with that of the uninephrectomized diabetic group.

Cobalt chloride preparation and treatment
Cobalt chloride hexahydrate (CoCl2.6H2O) was obtained from SD Fine Chemicals, Mumbai, India. Cobalt chloride hexahydrate is magenta colored, in a crystalline powdered form, with great solubility in normal saline. Based on a study and its LD50 value in rat by intraperitoneal route, a 10 mg/kg dose was selected for use in this study. Cobalt chloride hexahydrate solution was freshly prepared in normal saline to make a CoCl2 solution with a concentration 10 mg/mL. Animals were treated with cobalt chloride at the dose of 10 mg/kg, ip, OD for 30 days with continued dosing for one week, followed by intermittent dosing at days 9, 11, 13, 15, 17, 19, 21, 23, 25, 27 and 30. The animals in groups I and II were administered the equivalent amount of citrate buffer intraperitoneally as well as normal saline (1 mL/kg) intraperitoneally, and group III received normal saline (1 mL/kg, ip).

Statistical analysis
Results were expressed as mean±SEM. Results from each group at each period were compared with the respective value of that period of the control group. All comparisons were carried out on software Graphpad, PRISM®, version 5 using one-way analysis of variance (ANOVA) followed by a Tukey test and unpaired t-test, depending on the type of competition. P≤0.05 was considered statistically significant and P<0.001 highly significant.

Result
Effect of cobalt chloride treatment on general features of animals during the study
The unilateral nephrectomy followed by STZ administration induced moderate to severe CRF in rats. The biochemical parameters for their confirmation were measured as basal and after 48 h of STZ treatment and subsequently after 1, 3 and 5 weeks. CRF were significantly induced in the diabetic nephropathy model, while control animals, as well as sham operated animals, remained normal throughout the study period. A gradual decrease in the body weight of diabetic animals was observed with a significant effect observed at the fifth week (data not shown).

Effect of cobalt chloride treatment on plasma creatinine, urea and uric acid
Plasma creatinine, urea and uric acid were estimated in the control, uninephrectomized diabetic and CoCl2 groups. Animals in the uninephrectomized diabetic group showed a significant and stable rise in their plasma urea, creatinine and uric acid. A significant rise was observed in uninephrectomized diabetic group as compared to control after 48 h of STZ administration till the end of the 5th week at which a maximum difference was observed between these two groups. The animals which died during the study from the diabetic group also had a great increase in levels of renal biochemical parameters before their death (Figure 1).

Effect of cobalt chloride treatment on electrolyte levels
Plasma levels of sodium, potassium and chloride were measured. We observed a significant change in the electrolyte levels in group III. A significant decline in sodium level (135.55±6.36) was observed after 48 h of STZ administration which was normalized over the following week, after which a gradual decline in the sodium level was observed which was significant at the end of the 5th week. A rise in plasma potassium levels (6.60±0.35) was observed which was at its maximum at the end of the 3rd week and was differed significantly from the control group. There was a consistent fall in the plasma chloride levels in the uninephrectomized diabetic group (82.73±3.74) that was significantly different
from the control group (94.37±3.79). Treatment with cobalt chloride at the dose of 10 mg/kg, ip reduced the renal parameters, and partially normalized values were observed at the end of the study. A decline in animal mortality was also observed which was found in the diabetic group due to a worsening of renal function (Table 1).

Each value is represented as mean±S.E.M. (n=06). *P<0.05 vs control, °P<0.01 vs control, †P<0.001 vs control, ‡P<0.01 vs uninephrectomized diabetic.

Blood glucose level

Blood glucose level was estimated in plasma from control, uninephrectomized diabetic and CoCl₂ treatment groups at the start of the study, 48 h after STZ administration and at the end of the 5th week. No significant difference in basal values for glucose was observed in control or in uninephrectomized diabetic groups. But a significant rise was observed in the diabetic group after 48 h of STZ administration, with a persistent rise following thereafter; elevated plasma glucose levels were found in uninephrectomized diabetic group throughout the study. Treatment with CoCl₂ (10 mg/kg i.p.) decreased the plasma glucose level, although normalization of glucose was not observed in the treatment group. However, a significant difference was observed in plasma glucose levels in both groups (Figure 2).

Change in the dry contralateral kidney weight

Changes in dry weight of kidney of uninephrectomized diabetic and CoCl₂ group were estimated by comparing the weight of the kidney with that from an animal which had undergone a sham nephrectomy. A significant rise in the kidney weight was observed in diabetic group as compared to the sham group and a decrease in kidney weight was observed in CoCl₂ group (Figure 3).

Discussion

Although conventional treatments such as insulin and other antidiabetic drugs are used to reduce blood glucose levels and complications of diabetes, there is still a therapeutic need for effective drugs. Diabetic nephropathy is one of the complications of diabetes and involves progressive development of renal insufficiency in the setting of hyperglycemia. Diabetic nephropathy is now a major single cause of end-stage renal failure in many countries. Reliable animal models of diabetic renal injury may be a valuable tool for identifying the molecular mechanisms and for the pre-clinical development of new therapeutic strategies.

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Figure 1. Effect of cobalt chloride (10mg/kg i.p.) treatment for 30 days on biochemical parameters in control, uninephrectomized diabetic and CoCl₂ treatment group. (A) Plasma creatinine (mg/dL). (B) Plasma Uric acid (mg/dL) and (C) plasma uric acid (mg/dL). Each value is expressed as mean±S.E.M. (n=06). ***P<0.001 vs control, †P<0.05 vs uninephrectomized diabetic, **P<0.01 vs uninephrectomized diabetic, ***P<0.001 vs uninephrectomized diabetic.
Unilateral nephrectomy followed by streptozotocin (STZ) administration induced diabetes in rat and is a well documented model of experimental diabetes. Previous data show that the type of diabetes and characteristics differ with the dose of STZ employed and the animal species used. STZ is a pancreatic β cell toxin that induces rapid and irreversible necrosis of β cells of langerhans. Moreover, STZ-induced diabetes in rodents results in development of nephropathy similar to early stage clinical diabetic nephropathy, which accelerates the progression of renal injury. Uninephrectomy results in enlargement of the remaining kidney, further increased by the development of diabetes. A study demonstrated that uninephrectomy increases glomerular capillary pressure in SHR rats which promotes diabetic glomerular injury. In a study by Utimura et al., uninephrectomized (right nephrectomy) male wistar rats were made diabetic by a single intravenous injection of STZ (55 mg/kg) and blood glucose assessed two days later. The blood glucose was then maintained between 300-400 mg/dL for the next eight months with insulin treatment. We employed this model to induce diabetic nephropathy which eventually leads to chronic renal failure. An imbalance between oxygen supply and consumption disturbs local metabolism and leads to tissue hypoxia. There is compelling evidence to show that chronic hypoxia in the kidneys is the end result of multiple processes and mechanisms in patients with chronic renal disease. In spite of the fact that blood flow to the kidney is relatively high, the presence of oxygen shunt diffusion between arterial and venous vessels that run in close parallel approximation keeps renal tissue oxygen tension relatively low, suggesting hypoxia as one such determinant in the sensitivity of the kidney to changes in oxygen delivery. The hypoxia-inducible factor (HIF) is a heterodimeric nuclear factor, which is a crucial intermediate in the defense mechanisms against hypoxia, and its activation might offer a promising approach to the protection of hypoxic tissues by inducing a broad and coordinated downstream reactions. HIF is composed of two subunits, an oxygen-sensitive HIF-alpha subunit and a constitutively expressed HIF-beta subunit (also called ARNT, the aryl hydrocarbon receptor nuclear translocator). HIF stability is radically reduced by prolyl hydroxylases (PHDs) that induces oxygen-dependent hydroxylation of proline residues within the HIF proteins. The von Hippel Lindau tumor suppressor protein (pVHL) is then recruited by hydroxylated HIF, which in turn tags HIF with ubiquitin groups and targets it for degradation within the proteasome. However, under hypoxic conditions, HIF is not hydroxylated but is transactivated in the nucleus, activating a host of genes involved in the adaptation to hypoxia. Furthermore, HIF activation is suboptimal in renal disease and this strategic route is consequently wide open to discussion with a wide range of evidence from a variety of studies. Interestingly, cobalt inhibits HIF degradation by PHDs, thus enhancing HIF activity. A similar effect of cobalt has been previously demonstrated that treatment of STZ-induced diabetic rats with CoCl₂ results in a significant upregulation of HIF and HIF-regulated genes, and to a mitigated progression of renal failure in an obese, hypertensive type 2 diabetes rat model independent of metabolic status and blood pressure. The effect of CoCl₂ was attributed to the upregulation of HIF and HIF-regulated genes, and to a mitigated advanced glycation and oxidative stress. A similar effect of cobalt has been previously reported in other renal injury animal models. However, renoprotective mechanisms of cobalt remain elusive. Conversely, a study demonstrated that treatment of STZ-induced diabetic rats with CoCl₂ results in a significant upregulation of HIF and HIF-regulated genes, and to a mitigated progression of renal failure in an obese, hypertensive type 2 diabetes rat model independent of metabolic status and blood pressure. The effect of CoCl₂ was attributed to the upregulation of HIF and HIF-regulated genes, and to a mitigated advanced glycation and oxidative stress. A similar effect of cobalt has been previously reported in other renal injury animal models. However, renoprotective mechanisms of cobalt remain elusive.

### Table 1. Effect of cobalt chloride (10mg/kg, i.p.) treatment for 30 days on electrolyte levels.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Time period</th>
<th>Control group</th>
<th>Uninephrectomized diabetic group</th>
<th>CoCl₂ treatment group</th>
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<tr>
<td>Plasma Sodium (mMol/L)</td>
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<td>48 hrs after STZ</td>
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<td>1 week post treatment</td>
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<td>5 week post treatment</td>
<td>140.13±3.39</td>
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<td>Plasma Potassium (mMol/L)</td>
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<td>48 hrs after STZ</td>
<td>4.66±0.79</td>
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Figure 2. Effect of cobalt chloride (10mg/kg, i.p.) treatment for 30 days on blood glucose in control, uninephrectomized diabetic and CoCl₂ treatment group. Each value is expressed as mean±S.E.M. (n=06). *P<0.05 vs uninephrectomized diabetic, **P<0.001 vs control.

Figure 3. Effect of cobalt chloride (10mg/kg, i.p.) treatment for 30 days on controlateral kidney weight in sham, uninephrectomized diabetic and CoCl₂ treatment group. Each value is expressed as mean±S.E.M. (n=06). *P<0.05 vs control.
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References


15. Semenza GL. HIF-1, O(2), and the 3 PHDs: how animal cells signal hypoxia to the nucleus. Cell 2001;107:1-3.


