Original Article

Nephrotoxicity of Isosorbide Dinitrate and Cholestasis in Rat: The Possible Role of Nitric Oxide

A.M. Sharifi[1], N. Akbarloo[1], F. Sasani[2]

Abstract

Background: Nitric oxide (NO), a major chemical form of endothelium-derived relaxing factor and an important regulator of vascular tone, is released by endothelial cells. The role of NO is not restricted to the vascular system, and it participates in the regulation of renal hemodynamics and renal excretory function. There are increasing evidences indicating that the elevated levels of NO play a primary pathogenic role in the glomerular injury and renal failure.

Objectives: We sought to investigate the renoprotective or nephrotoxic effects of various doses of isosorbide dinitrate as an exogenous model, and induction of cholestasis as an endogenous model of NO overproduction on renal function and structure.

Methods: Parameters such as plasma and urine p-nitrophenyl-N-acetyl-β-D-glucosaminidase (NAG)-activity, urea and creatinine levels were measured.

Results: Urea, creatinine and NAG-activity in rats treated by different doses of isosorbide as well as in cholestasis induced rats, were higher than control group. This elevation was significantly pronounced at higher doses of isosorbide.

Conclusion: NO overproduction would be nephrotoxic due to oxidizing products of NO, peroxynitrite anion ($ONOO^-$), formed by the reaction of NO and superoxide radical (O_2^-).
Iran J Med Sci 2004; 29(2):75-80.

Keywords • Nitric oxide • cholestasis • nephrotoxicity

[1]Department of Pharmacology, School of Medicine, Endocrinology and Metabolism Research Center (EMRC), Dr Shariati Hospital, Tehran University of Medical Sciences,

[2]Department of Pathology, Veterinary School, Tehran University, Tehran, Iran

Correspondence: Ali Mohammad Sharifi, PhD
Department of Pharmacology,
School of Medicine,
Iran University of Medical Sciences,
P.O.Box 14155-6183,
Tehran, Iran

Tel: +98 21 805 86 96
Fax: +98 21 805 8719
E-mail: sharifal@yahoo.com

Introduction

Nitric oxide (NO) is a major chemical form of endothelium-derived relaxing factor,[1] and is an important regulator of vascular tone released by endothelial cells.[2] The effects of NO on various cells are associated with activation of soluble guanylate cyclase and subsequent elevation of intracellular cGMP. The role of NO is not restricted to the vascular system. Alteration in NO synthesis has been incriminated in several other pathophysiological conditions, including arterial hypertension, progression of renal failure, arteriosclerosis, septic shock, and neurodegerative disorders.[3,4] The extended research in the past years revealed the large involvement of L arginine-NO pathway in the renal physiology. NO participates in the regulation of renal hemodynamic and renal

Table1: 24-hr urine volume and mean arterial blood pressure of isosorbide dinitrate treated and cholestatic rats.								
Group	Saline	Isosorbide dinitrate (mg/kg)					SHAM	BDR
		5	10	15	20	25		
UV	37±1.2	36±0.8	32±1.9	27±1.1*	19±0.98**	14±0.87**	35±1.1	20±1.5++
MBP	108±4.1	105±3.3	106±4.9	103±3	104±5.2	100±3.0	95±2.4	101±4.5

Data are presented as Mean±SEM for each group (n=8). UV= urinary volume (ml/24 hr), MBP= Mean Blood Pressure (mm Hg), BDR=Bile Duct Resected, SHAM= sham operated.
*$p < 0.05$ and **$p < 0.01$ *vs.* saline. ++$p < 0.01$ *vs.* SHAM.

excretory functions under basal and stimulated conditions.[5,6] NO is synthesized in the kidney from L-arginine by both the constitutive, calmodulin-dependent NO synthase (cNOS) and inducible NOS (iNOS).[7] The cNOS is synthesized in the cells of endothelium of glomerular vessels, vasa recta, tubular epithelium and the inner modulary-collecting duct and the iNOS is expressed in the mesangial and tubular cells.[4,8,9] Controversial evidences exist about the role of NO-cGMP pathway in pathophysiology of renal disease. Many investigators believe that NO has a renoprotective role by maintaining renal vasodilatation and inhibiting platelet adhesion and aggregation.[10] On the other hand, numerous evidences exist indicating that the elevated levels of NO may play a primary pathogenic role in some form of glomerular injury and renal failure.[4] It is shown that radiocontrast agents, substances that are largely used in diagnostic procedures, are presented with nephrotoxicity, as their major side effects, mainly due to the decreased NO production.[11] Excessive or overproduction of NO, as seen in septic shock, are also thought to be cytotoxic by several mechanisms, including production of free radicals, formation of peroxynitrite, nitrosylation, and therefore, inactivation of various enzymes.[3,12]

Cholestasis or impaired hepatocellular secretion of bile is a complication of progressive liver disease. Recent reports have suggested that high liver NO production might be an important mediator of cholestasis, which may result in hyperdynamic circulation observed in cirrhosis and impairment of cGMP-associated vasodilatation.[13,14] The measurement of N-acetyl-B-D-Glucosaminidase activity (NAG-activity) is a well-known, sensitive and reliable method for assessing nephrotoxicity.[15,16] In the present study the role of isosorbide dinitrate (ID) administration, an exogenous NO producing model, and cholestasis, an endogenous NO overproduction model, on renal function and structure are investigated.
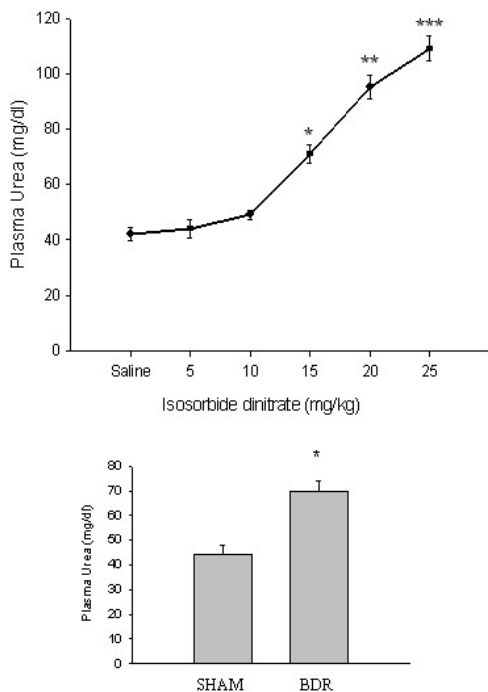
Materials and Methods

Sixty-four male Sprague-Dawley rats (200–250 g) were randomly divided into eight groups of equal size as described bellow, and housed in standard rat cages at room temperature of 20–25 °C, under a 12 hr dark/light cycle and had free access to water and standard rat chew.

Experimental Design
Group 1 received saline as vehicle and considered as control group. Groups 2 to 6 received a solution of isosorbide dinitrate (Sigma, USA) at doses of 5, 10, 15, 20, and 25 mg/kg by gavages, for 10 consecutive days. At day 11, under light ether anesthesia, arterial blood pressure was measured by tail-cuff plethysmography. Then the animal was placed in a metabolic cage (Razi Research Center) for the collection of 24-hr urine output and the determination of urine NAG-activity. Then, the animal was anesthetized with sodium pentobarbital (50 mg/kg ip) to take 5 ml blood sample from tail vein for the measurement of plasma NAG-activity, plasma urea and creatinine concentrations.

Indication of cholestasis
General anesthesia was induced by sodium pentobarbital (50 mg/kg, ip) in groups 7 and 8. In group 7 the sham-operated group after opening the abdominal cavity, bile duct was identified, manipulated and left *in situ* without being resected. In group 8 the cholestatic group the surgery was done as in group 7, but the bile duct was resected, as described by Bergasa and collogues.[17] After closing the wounds, each animal received 0.4 ml of normal saline subcutaneously and then placed in separate cages for the prevention of wound dehiscence. Seven days after the surgery, each animal was placed in a metabolic cage to collect 24-hr urine output. Then the animal was anesthetized to collect blood samples as mentioned above.

A.M. Sharifi, N. Akbarloo, F. Sasani

Fig 1: Top: Effects of different doses of isosorbide dinitrate on plasma urea. Bottom: Alteration of plasma urea in BDR and SHAM operated rats. Results are given as mean and vertical bars indicate SEM. *p<0.05, **p<0.01, ***p<0.001 vs. respective control groups.

Fig 2: Top: Effect of different doses of isosorbide dinitrate on plasma creatinine. Bottom: Alteration of plasma creatinine in bile duct resected (BDR) and sham operated (SHAM) rats. Results are given as mean and vertical bars indicate SEM. *p<0.05, **p<0.01, ***p<0.001 vs. respective control groups.
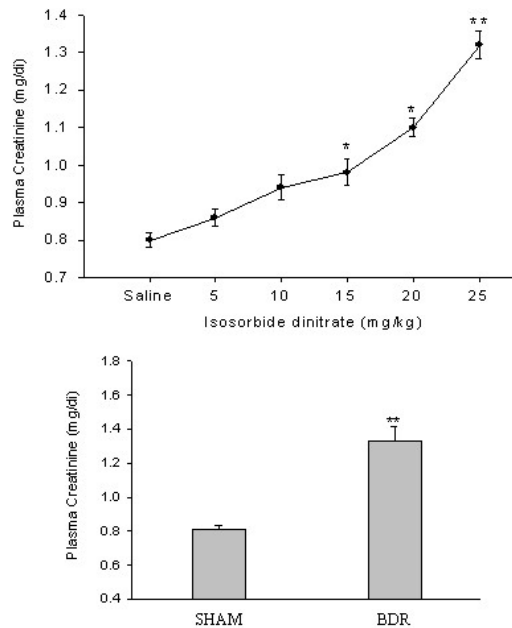
Analytical Methods
Assay for the measurement of NAG-activity is based on the enzymatic hydrolysis of p-nitrophenyl-N-acetyl-β-D-Glucosaminidase (PNP-NAG; Fluka, UK) at pH 4.4 and spectrophotometry of the liberated para-nitrophenyl (PNP) at wavvlengh of 405 nm.[18] In this study, the most reliable results were provided at sample/reagent volume ratios of 1/6 as reported by other investigators too.[19]

Enzymatic Reactions
Enzyme activity was evaluated as stated by Noto and his colleagues.[20] In brief, two labeled test tubes, urine (U) and urine blank (UB) were used, respectively. Each test tube was filled with 0.5 ml plus 0.5 ml NAG-substrate solution, p-nitrophenyl (5 mM; Sigma, USA) and incubated for 2 hrs at 37 °C. After 2 hrs,The reaction terminated by adding 2 ml of 0.05 M sodium carbonate-bicarbonate buffer (pH 10.7). In this procedure, one unit of enzyme catalyzed the formation of 1 µM of PNP.

Histological Evaluation of the Kidney
At the end of the experiment and under deep anesthesia, the kidneys were dissected, cleansed and placed in 10% buffered formalin. The specimens of renal tissues, then were prepared, blotted and stained with H/E method and studied under light microscopy.
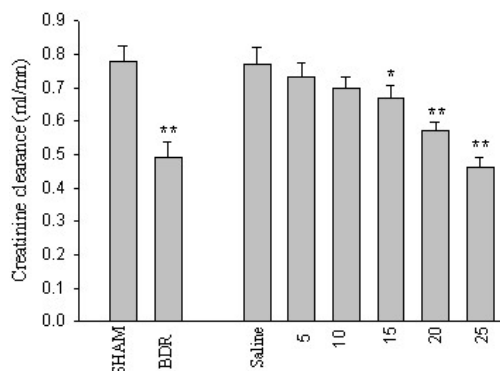
Statistical Analysis
All values are expressed as mean±SEM. *Student's t*-test was used for statistical analysis and the differences were considered significant at p<0.05.
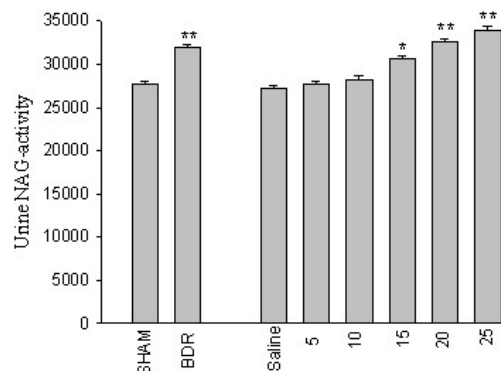
Results

Administration of various doses of ID or induction of cholestasis did not a profoundly influence mean arterial blood pressure (MBP) whereas, a marked oliguria was observed in both ID and BDR groups (Table 1).

BUN and plasma creatinine of ID groups increased steadily in a dose-dependent manner, becoming significant at doses of 15, 20 and 25 mg/kg (Fig 1). These increments were accompanied with a concomitant and marked reduction in creatinine clearance (Fig 3). Cholestasis also markedly increased BUN and plasma creatinine with a concomitant reduction of creatinine clearance (Figs 1, 2 and 3).

A significant elevation of urine NAG-activity was observed in ID administered rats with doses of 15, 20 and 25 mg/kg and also in BDR group (Fig 4).

77

Fig 3: Creatinine clearance in isosorbide dinitrate (mg/kg), bile duct resected (BDR) and control groups. Data are as mean and vertical bars indicate SEM. *p<0.05, **p<0.01 vs. their respective control groups.

Fig 4: Urine NAG-activity in isosorbide dinitrate (mg/kg), BDR and control groups. Data are mean and vertical bars indicate SEM. *p<0.05, **p<0.01 vs. their respective control groups.

Discussion

Nephrotoxicity is a major side effect of drug therapy in clinical practice, frequently leading to acute renal failure (ARF). Many physiological mechanisms have been implicated in drug-induced renal injury. Currently, NO is considered to be an important regulator of renal vascular tone and a modulator of glomerular functions under both physiological and pathological conditions. Historically, NO has been implicated in ARF and, after its discovery, several publications have suggested that changes in NO production could play an important role in the hemodynamic alterations that observed in ARF.[21] Earlier reports have indicated that NO, as an emerging molecule, may play an important role in renal physiology and pathophysiology and the L-arginine–NO pathway is important for the prevention, as well as the treatment, of many forms of renal diseases. The cNOS is produced in the endothelial cells of glomerular vessels and vasa recta and also in the tubular epithelial cells. The iNOS is expressed in the mesengial and tubular cells.[5,8,12] These enzymes that are widely distributed in the kidney produce NO from L-arginine.[22]

The results of the present study supported the hypothesis that overproduction of NO might be nephrotoxic. In order to evaluate this hypothesis, two models of NO overproduction are used in the rat. In exogenous NO overproduction model we chronically administered isosorbide dinitrate at various doses for 10 days, and endogenous NO overproduction model, we induced cholestasis as described by other investigators.[13,14] As shown in Table 1, cholestasis and administration of ID caused oliguria and reduced urine volume in a dose-dependent manner with a concomitant reduc-

tion in urine output. As shown in Figs 1, 2, and 4, all measured plasma parameters urea, creatinine, and NAG-activities were significantly higher in both ID and BDR groups.

There is compelling evidence on both beneficial, as well as detrimental role of NO in the kidney. Several studies have demonstrated that stimulation of NO synthesis by administration of exogenous L-arginine attenuated the reduced glomerular filtration rate (GFR) induced by renal ischemia.[23,24] It has been assumed that much of the beneficial effects of NO is to maintain or restore renal blood flow. Schramm and colleagues showed that NO has also a beneficial effect on systemic and pulmonary hemodynamics in the setting of renal ischemia.[24]

Moreover, Yu *et al.* demonstrated a detrimental effect of NO on freshly isolated rat proximal tubules exposed to hypoxia and reoxygenation.[4] In their experiments, No-synthase inhibitors have shown to have a protective role against renal injury, whereas administration of NO donors, such as sodium nitroprusside, and enhanced hypoxia/ reoxygenation injury.[4] Paller and coworkers confirmed the role of NO in hypoxia injury of renal tubular epithelium.[1] Noiri *et al,* demonstrated that during renal ischemia iNOS had a toxic effect on the kidney whereas, its inhibition improved renal functions.[31] The most potent oxidizing product of NO is peroxynitrite ($ONOO^-$) anion, formed by the reaction of NO with superoxide (O_2^-) radical.[25,26] This molecule is a potent sulfhydryl-oxidizing compound. It is easily protonated to an unstable product of peroxynitrous acid (ONOOH), that spontaneously forms hydroxyl radical (OH^-) and nitrogen dioxide (NO_2^-), or may undergo an intramolecular rearrangement to yield nitrate (NO_3^-).[27] Therefore, NO may exert its potential toxic effect

through the formation of peroxynitrite and/or its by products to initiate lipid peroxidation and intracellular injury.[6,10]

There are numerous reports about the direct cytotoxicity of NO that are consistent with our findings.[4,5] Recent investigations have claimed that NO is toxic to many cell types.[10] Neutrophils, natural killer cells and activated macrophages kill the bacteria using NO as a defense mechanism.[26-29] Neuronal cells die via induction of apoptosis in the presence of NO or peroxynitrite.[30] Therefore, these results lead us to conclude that NO not only has a key role in physiological regulation of renal blood flow and glomerular hemodynamics but at the same time its overproduction may damage renal cells. Further studies are needed to conclude that the inhibition of NO synthase may prevent renal toxicity in cholestatic or isosorbide treated animals.

Conclusion

In conclusion, it seems that NO may exert its potential nephrotoxic effect through the formation of peroxynitrite to initiate lipid peroxidation and cellular injury.

Acknowledgments

References

1 Paller MS. Nitric oxide-mediated renal epithelial cell injury during hypoxia and re-oxygenation. *Ren Fail* 1998; **20**: 459-9.

2 Furchgott RF, Zawadzki JV. The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. *Nature* 1980; **288**: 373-6.

3 Baylis C, Bloch J. Nitric oxide (NO) in renal physiology and pathophysiology. *Nephrol Dial Transplant* 1996*;* **11**: 1955-8.

4 Yu L. Role of Nitric oxide in acute renal failure. *Renal Failure* 1997; **19**: 213-6.

5 MacAllister R, Vallance P. Nitric oxide in essential and renal hypertension. J. Am. Soc. *Nephrol* 1994; **5**: 1057-65

6 Radi R, Beckman JS, Freeman BA. Peroxynitrite-induced membrane lipid peroxidation: the cytotoxic potential of superoxide and nitric oxide. *Arch. Biochem. Biophys* 1991; **288**: 481-7.

7 Forsterman U, Pollock JS, Nakane M. Nitric oxide synthesis in the cardiovascular system. *Trends. Cardiovasc Med* 1993; **3**: 104-10.

8 Morrissey JJ, McCracken R, Kaneto H, Vehaskari M. Location of an inducible nitric oxide synthase mRNA in the normal kidney. *Kidney Int* 1994; **45**: 998-1005.

9 Shultz PJ, Archier SL, Rosenberg ME. Inducible nitric oxide synthase mRNA and activity in glomerular mesangial cells. *Kidney Int* 1994; **46**: 683-9.

10 Raij L, Jaimes D, Castillo D, et al. Pathophysiology of the vascular wall: The role of nitric oxide in renal disease, Prostaglandins. *Leukotriens and Essential Fatty Acids* 1996; **54**: 53-8.

11 Ribeiro L, de Assuncao E, Silva F, et al. Evaluation of the nitric oxide production in rat renal artery smooth muscle cells culture exposed to radiocontrast agents. *Kidney International* 2004; **65**: 589-96.

12 Raij L, Baylis C. Glomerular actions of nitric oxide. *Kidney Int* 1995; **48**: 20-32.

13 Abrams GA, Trauner M, Nathanson MH: Nitric oxide and liver disease. *Gastroentrologist* 1995; 3: 220-3.

14 Inan M, Sayek I, Tel BC, Sahin I. Role of endotoxin and nitric oxide in the pathogenesis of renal failure in obstructive jaundice. *Br. J. Surg* 1997; **84**: 943-7.

15 Garvey MJ, Tuason VB, Hwang S. Use of renal enzymes to evaluate nephrotoxicity in lithium treated patients. *Br. J. Psychiat* 1982; **141**: 420-2.

16 Lim CW. N-acetyl-B-D-glucosaminidase assay by the p-nitro-phenol-technique: Inhibitory effects of urine as decreased by gel filtration and by simple dilution. *Clin Chem* 1987; **33**: 713-4.

17 Bergasa, NV, Rothman RB, Vergalla H, et al. Central mu-opioid receptors are down-regulated in a rat model of cholestasis**.** *J Hepatol* 1992; **15**: 220-4.

18 Horak E, Hopfer SE, Sunderman FW. Spectrophotometric assay for urinary N-acetyl-β-D-glucosaminidase activity. *Clin Chem* 1981; **27**: 1180-5.

19 Jung K, Klotz S. More about determination of N-acetyl-B-D-glucosaminidase activity in urine without pretreatment of samples. *Clin. Chem* 1988; **34:** 1002-6.

20 Noto A, Morita A, Numata Y, Kosugi Y. Stabilities of N-acetyl- beta- D-glucosaminidase (NAG) isoenzymes in urine: advantage of NAG isoenzyme B measurement in clinical applications. *Clin. Chim. Acta* 1998; **278**: 35-43.

21 Valdivielso JM, Blantz RC. Acute renal failure: is nitric oxide the bad guy? Antioxid Redox Signal 2002; **4**: 925-34.

22 Peer G, Blum M, Iaina A. Nitric oxide and acute renal failure. *Nephron* 1996; **73**: 375-

81.

23 Dagher F, Pollina RM, Rogers DM, et al. The value and limitations of L-arginine infusion on glomerular and tubular function in the ischemic/reperfused kidney. *J Vasc Surg* 1995; **21**: 453-8.

24 Schramm L, Heidbreder E, Schmitt A, Heidland. A Role of L-arginine-drived NO in ischemic acute renal failure in the rat. *Renal Failure* 1994; **16**: 555-9.

25 Saad SY, Najjar TA, Daba MH, Al-Rikabi AC. Inhibition of nitric oxide synthase aggravates cisplatin-induced nephrotoxicity: effect of 2-amino-4-methylpyridine. *Chemotherapy* 2002; **48**: 309-15.

26 Koppenol WH, Moreno JJ, Prior WA, Beckman JS. Peroxynitrite, a cloaked oxidant formed by nitric oxide and superoxide, *Chem. Res. Toxicol* 1992; **5**: 834-42.

27 Beckman JS, Beckman TW, Marshall PA, Freeman BA. Apparent hydroxyl radical production by peroxynitrites implications for endothelial injury from nitric oxide and superoxide. Proc. Natl. Acad. Sci. USA 1990; **87**: 1620-4.

28 Xiao L, Eneroth PH, Qureshi GA. Nitric oxide synthase pathway may mediate human natural killer cell cytotoxicity. Scand. *J. Immunol* 1995; **42**: 505-11.

29 Pacelli R, Wink DA, Cook JA, and Mitchell JB. Nitric oxide potentiates hydrogen peroxide-induced killing of Escherichia coli. *J. Exp. Med* 1995; **182**: 1469-79.

30 Estevez AG, Radi R., Barbeito L, Beckman JS. Peroxynitrite-induced cytotoxicity in PC12 cells, evidence for an apoptotic mechanism differentially modulated by neutrophic factors. J. *Neurochem* 1995; **65**: 1543-50.

31 Noiri E, Peresleni T, Miller F, Goligorsky MS. In vivo targeting of inducible NO synthase with oligodeoxynucleotides protects rat kidney against ischemia. *J Clin Invest* 1996; **97**: 2377-83.