**Alpha-2 Adrenergic Agonists Protect Against Radiocontrast-Induced Nephropathy in Mice.**

F. T. Billings, IV, MD\(^1\), Sean W. C. Chen, BS\(^1\), Mihwa Kim, BS\(^1\), Sang Won Park, PhD\(^1\), Joseph H. Song, BS\(^1\), Shuang Wang, PhD\(^2\), Joseph Herman, PhD\(^4\), Vivette D’Agati, MD\(^3\), and H. Thomas Lee, MD, PhD\(^1\,\#\)

Departments of \(^1\)Anesthesiology, \(^2\)Biostatistics, and \(^3\)Pathology, College of Physicians and Surgeons of Columbia University, New York, NY 10032 and \(^4\)Division of Clinical Pharmacology, The Children’s Hospital of Philadelphia, Philadelphia, PA 19104.

**Running Title:**
Alpha-2 agonists reduce radiocontrast nephropathy

**Address for Correspondence:**
H. Thomas Lee, M.D., Ph.D.
Associate Professor
Department of Anesthesiology
Anesthesiology Research Laboratories
Columbia University
P&S Box 46 (PH-5)
630 West 168\(^{th}\) Street
New York, NY 10032-3784
Tel: (212) 305-7416 (Office)
    (212) 305-1807 (Lab)
Fax: (212) 305-8980
E.Mail: tl128@columbia.edu

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**Key Words:** acute renal failure, iohexol, clonidine, dexmedetomidine, yohimbine, HK-2 cells, medullary ischemia
Abstract

Radiocontrast nephropathy (RCN) is a common clinical problem without effective therapy. Utilizing a murine model, we tested the hypothesis that alpha-2 adrenergic receptor agonists [clonidine, dexmedetomidine (dex)] protect against RCN, induced with iohexol (a nonionic low-osmolar radiocontrast). C57BL6 mice were pretreated with saline, clonidine, or dex before induction of RCN. Some mice were pretreated with yohimbine (a selective alpha-2 receptor antagonist) prior to saline, clonidine or dex administration. Alpha-2 agonist treated mice had reduced plasma creatinine, renal tubular necrosis, renal apoptosis, and renal cortical proximal tubule vacuolization 24 hrs after iohexol injection. Yohimbine reversed the protective effects of both clonidine and dex pretreatment. Injection of iohexol resulted in a rapid (~90 min.) fall of renal outer-medullary blood flow. Clonidine and dex pretreatment significantly attenuated this perfusion decrease without any changes in systemic blood pressure. To determine whether proximal tubular alpha-2 adrenergic receptors mediate the cytoprotective effects, we treated cultured human proximal tubule (HK-2) and rat pulmonary microvascular endothelial cells (RPMEC) with iohexol after vehicle, clonidine or dex pretreatment. Iohexol caused a direct dose-dependent reduction in HK-2 and RPMEC viability, but alpha-2 agonists failed to preserve the viability of both cell types. We conclude that alpha-2 adrenergic receptor agonists protect mice against RCN by preserving outer-medullary renal blood flow. As alpha-2 agonists are widely utilized during the perioperative period, our findings may have significant clinical relevance to improving outcomes following radiocontrast exposure.
**Introduction**

Arteriography, angiocardiography, and contrast enhanced CAT scans, account for more than 3 million iodinated radiocontrast exposures each year in the U.S. (15). Renal dysfunction secondary to radiocontrast administration remains prevalent and debilitating (3). Radiocontrast nephropathy (RCN) remains the third most common cause of inpatient acute renal failure behind ischemia reperfusion injury and nephrotoxic medication administration (23). The diagnosis of RCN confers a 5.5 fold increase in hospital mortality (18, 29), may necessitate hemodialysis (7), and is associated with an increased hospital length of stay and incidence of myocardial infarction (25).

Although the pathogenesis of RCN remains incompletely understood, tubular hypoxic injury, due to a reduction in renal medullary blood flow, and direct tubular cytotoxicity play a substantial role (8, 12, 31). The risk of developing nephropathy following radiocontrast exposure may be as high as 50%, depending on numerous risk factors (21). Pre-existing renal dysfunction and dehydration are the most predictive contributors to RCN, while volume of contrast exposure, contrast osmolality, congestive heart failure, diabetes, anemia, and advanced age also increase risk (2, 3). Despite the exploration of numerous prophylactic regiments (N-acetylcysteine, theophylline, sodium bicarbonate, dopamine, fenoldopam, calcium channel blockers) and attempts at developing less toxic (lower osmolar) contrast agents, isotonic intravenous hydration remains the only proven RCN prophylaxis (3, 22, 30).

Alpha-2 agonists have diuretic (26) and sympatholytic effects (20). Having noted the corticomedullary ischemic pathogenesis of radiocontrast-induced nephropathy and the decreased systemic vascular resistance and diuretic properties of alpha-2 agonism, we
hypothesized that exogenously administered alpha-2 agonists would preserve outer-medullary renal blood flow and protect against renal dysfunction following iodinated radiocontrast exposure. To test this hypothesis, we utilized both *in vivo* and *in vitro* models of RCN.
**Materials and Methods**

**Mice**

Male C57BL/6 mice (25 grams) were purchased from Harlan Laboratories (Indianapolis, IN). The Columbia University Institutional Animal Care and Use Committee approved the animal care protocol for the experiments performed in this study.

**Murine model of radiocontrast-induced nephropathy**

Radiocontrast nephropathy (RCN) was induced in mice as described previously (16). After overnight (16 hr) water deprivation and prior inhibition of prostaglandin and nitric oxide synthesis, mice were injected subcutaneously (SQ) with the low-osmolar monomeric iodinated radiocontrast media, iohexol (Omnipaque, 1.5 g iodine/kg). To inhibit cyclooxygenase and nitric oxide synthase, mice were injected with indomethacin 10 mg/kg SQ (dissolved in dimethylsulfoxide) and levo-nitroargininemethylester (L-NAME) 10 mg/kg SQ (dissolved in 0.9% saline), respectively, 15 minutes prior to iohexol injection. This model reliably produces nephropathy following radiocontrast injection, and has been previously validated in mice and rats (6, 16). Animals were then given access to food and water and sacrificed 24 h later for serum creatinine determination and kidney removal. Sham mice received SQ injections of saline instead of iohexol after indomethacin and \(N^G\)-nitro-\(l\)-arginine methyl ester injection and 16 hrs of water deprivation.

To determine whether alpha-2 adrenergic receptor agonist protect against murine RCN, we subjected mice to the following treatment groups: 1) saline 100\(\mu\)l bolus SQ plus
SQ infusion at 1μl/hr, 2) 5 or 10 μg/kg bolus clonidine SQ, 3) 5 or 10 μg/kg bolus clonidine SQ plus 5 or 10μg/kg/h infusion SQ, 4) 3μg/kg bolus dex SQ, 5) 3μg/kg bolus dex SQ plus 2 or 4μg/kg/h infusion SQ. Subcutaneous infusion of drugs or saline was achieved by micro-osmotic pumps (Alzet Co., Cupertino, CA; Model 1003D; 1 μl/hr), implanted 16 hrs prior to iohexol injection. To determine whether blockade of alpha-2 receptors prevents any dex- or clonidine-mediated protection against RCN, some mice were injected with 0.1 mg/kg yohimbine (a selective alpha-2 adrenergic receptor antagonist) after miniosmotic pump placement and before bolus injection of saline, clonidine, or dex.

Assessment of nephropathy after iohexol injection

We assessed renal function 24 hr after radiocontrast injection by determining plasma creatinine concentration, using the colorimetric method based on the Jaffe reaction (11), and by assessing kidneys for necrosis, apoptosis and cortical vacuolization (osmotic nephrosis).

For histological light microscopy preparations, we selected kidneys from six randomly selected mice from the saline RCN, clonidine (10ug/kg/h + 10ug/kg) RCN, and dex (4ug/kg/h + 3ug/kg) RCN groups, 24 hr after iohexol injection (following blood collection). Kidneys were bisected along their long axis and fixed in 10% formalin for 24 hours. Following automated dehydration through a graded alcohol series, transverse kidney specimens were imbedded in paraffin, sectioned at 5 μm, and stained with haematoxylin and eosin. An experienced renal pathologist, blinded to the animal treatment group, assessed proximal tubular necrosis, apoptosis, and cortical tubule
vacuolization. Tubular necrosis was quantified as the number of necrotic tubules per field (200X). At least 25 to 30 tubules were counted in each field, and six fields were examined for each slide.

Renal tubular apoptosis was qualitatively assessed by in situ Terminal Deoxynucleotidyl Transferase Biotin-dUTP Nick End Labeling (TUNEL) staining, which detects the DNA fragmentation characteristic of apoptosis. Fixed mouse kidney sections, obtained 24 hrs after iohexol injection, were deparaffinized in xylene and rehydrated through graded ethanols to water. In situ labeling of fragmented DNA was performed with TUNEL (green fluorescence) using a commercially available in situ cell death detection kit (Roche, Indianapolis, IN) according to the manufacturer's instruction. To visualize the total number of cells in the field, kidney sections were also stained with Hoeschst 33342 (blue fluorescence). The sections were quantified blindly by counting the labeled cells in 100 X magnified fields.

Quantification of plasma concentrations of clonidine and dex at time of radiocontrast injection

In order to measure the plasma concentration of alpha-2 agonists (clonidine or dex), we collected plasma 16 hrs after the placement of drug infusion micro-osmotic pumps. Plasma concentrations of clonidine and dex were quantified by solid-phase extraction and high pressure liquid chromatography before analysis with tandem mass spectroscopy (Children’s Hospital of Philadelphia, Philadelphia, PA), as previously described (17).
Assessment of outer-medullary renal blood flow after iohexol injection

In a separate cohort of male C57BL/6 mice, outer-medullary blood flow was recorded for 90 minutes following iohexol injection in our murine model of RCN. Systemic blood pressure was also recorded during this period by carotid artery cannulation. Outer medullary renal blood flow was quantified using laser-Doppler flow probes as described previously (1). Laser-Doppler flow probes provide reliable measurements of relative change in regional blood flow in rodent kidney (27). In brief, a needle flow probe (480 µm diameter; Model TSD145) connected to a laser-Doppler flow-meter (Biopac Systems, Goleta, CA) measures red blood cell volume and velocity in the cubic millimeter 1 mm distal to the tip of the flow probe. Flow is derived as the product of red blood cell volume and velocity. The relative change in outer medullary blood flow before and after radiocontrast injection was measured in saline, clonidine (10 μg/kg), and dexmedetomidine (3 μg/kg) pretreated mice.

Following 16 hr overnight dehydration, C57BL/6 mice were anesthetized with intraperitoneal pentobarbital (i.p.) (50 mg/kg or to effect) and placed them in a heating pad to maintain body temperature between 36 and 38°C. Additional pentobarbital (10% of the original dose) was given as needed, based on tail pinch. Right carotid artery was cannulated for blood pressure measurements, and we exposed the left kidney by laparotomy. The needle tip of the flow probe was then inserted directly into the renal cortex and advanced into the outer-medulla (approximately 1.5 mm beneath the surface of the kidney). Although the insertion of the probe is invasive, blood flow is measured in the undisturbed region 1 mm beneath the tip of the optical probe. Voltage output was recorded on a computer connected to a Biopac data acquisition system and displayed as
blood perfusion units. The flow data is then represented as the percent of the baseline blood flow for each mouse.

Baseline renal blood flow and systemic blood pressure were established prior to i.p. administration of saline (100μl), clonidine (10μg/kg), or dex (3μg/kg). Fifteen minutes later, indomethacin (10mg/kg) and l-NAME (10mg/kg) were injected i.p. Iohexol (1.5g iodine/kg) was then injected i.p. 15 minutes later. Medullary blood flow and systemic blood pressure were continuously recorded for 90 minutes after iohexol injection. At the end of each experiment, the mouse was sacrificed, and the kidney removed and bisected to confirm the position of the needle probe tip in the outer medulla. Mice with incorrectly placed probes were excluded from the study.

Cell Culture

Immortalized human proximal tubule cells (HK-2 cells, American Type Culture Collection, Manassas, VA) were grown and passaged in culture medium (50:50 mixture of low-glucose DMEM and F-12 plus 5% serum) and antibiotics (100 U/ml of penicillin G, 100 μg/ml of streptomycin, and 0.25 μg/ml of amphotericin B) at 37°C in a 100% humidified atmosphere of 5% CO₂ - 95% air. Rat pulmonary microvascular endothelial cells (RPMEC) were obtained from Dr. E. Heidi Jerome (Departments of Anesthesiology and Pediatrics, Columbia University) and grown in F-12 medium plus 10% serum. Cells were plated in 24-well plates and used in the experiments described below when confluent.

**In vitro models of radiocontrast nephropathy**
An *in vitro* model of radiocontrast injury was used as described previously (13, 16). Confluent monolayers of HK-2 cells or RPMEC were pretreated with saline, clonidine (10μM), or dex (10μM) for 30 minutes before iohexol (0, 50, 100, or 150mg/ml iodine) treatment. Following 16 hours of radiocontrast exposure, HK-2 cell viability was quantified by MTT assay.

**Measurement of cell viability**

Cell viability was assessed and quantified using a 3-[4,5-dimethyl(thiazol-2-yl)-3,5-diphenyl] tetrazium bromide (MTT) cytotoxicity assay as described previously [22]. The MTT assay measures mitochondrial dehydrogenase activity, a component of the Krebs cycle, and a marker of energy production and cellular viability. Spectrophotometry quantifies the formation of the dark blue formazan product of the reduction of MTT’s tetrazolium ring by mitochondrial dehydrogenase. A MTT tetrazolium salt solution was prepared fresh in serum-free medium at a final concentration of 0.5mg MTT/ml. After removal of cell plate media, 0.5 ml of this MTT solution was added to each well of saline, clonidine, or dex pretreated and iohexol treated cells, and incubated for 3 hours. This media was then removed. The MTT dark blue formazan was then solubilized and extracted by adding 0.5 mL of 0.05 M HCL in isopropanol to each well. After 15 minutes at room temperature, the optical densities of the formazan extracts of each well were quantified at 570 nm using a spectrophotometer and the results were expressed as the percentage of vehicle-treated (saline pretreated, 0mg/ml iohexol treated) cells.
Materials

Iohexol was obtained from Amersham Health (Princeton, NJ). Clonidine and yohimbine were obtained from Tocris (Ellisville, MO), dex from Hospira (Lake Forest, IL). All other reagents were obtained from Sigma (St. Louis, MO).

Statistical Analysis

Continuous variables are summarized throughout the text by their mean ±SE. When comparing plasma creatinine concentrations, necrotic tubules/hpf, and viability of HK-2 cells, data were analyzed using Student’s t-test. For preliminary analysis of outer-medullary cortical renal blood flow and systemic blood pressure, following radiocontrast exposure, we used the Student’s t-test at each time point. For better assessment of the treatment effect, we then used the linear mixed effect model to analyze outer-medullary renal blood flow and systemic blood pressure as longitudinal repeated measurements. During this comparison and assessment for differences between outer-medullary renal blood flow (or systemic blood pressure) profiles in clonidine and saline treated animals (or dex and saline treated animals), fixed effects included treatment, time from radiocontrast exposure, and treatment by time interaction. To account for the correlations between repeated measurements at different time points, mice were modeled using random effect. If the treatment by time interaction was statistically significant, the two profiles differ. That is, the treatment effect differs at different time points. If the treatment by time interaction was not statistically significant, it suggests that the two profiles are parallel and the treatment effect is the same at all time points. In this case, difference in profiles is then assessed using overall effect analysis.
P values less than 0.05 were considered statistically significant. We used SAS version 9.1 (SAS Institute, Cary, NC) for all statistical analyses.
Results

Development of radiocontrast nephropathy in mice

Sham mice (with prostanoid and nitric oxide synthesis inhibition but without ioxehol injection) had a plasma creatinine (Cr) concentration of 0.4±0.1 mg/dl, n=4. C57BL/6 mice treated with iohexol (1.5 g iodine/kg) after prostanoid and nitric oxide depletion developed acute renal failure (Cr=1.5±0.2 mg/dl, n=8, p<0.001 vs. sham mice, 24 hrs after iohexol injection). Our previous studies demonstrated that without prostanoid and nitric oxide inhibition, iohexol-injected mice do not develop RCN (16). Activation of alpha-2 adrenergic receptors with clonidine or dex prior to iohexol injection protected mice against RCN (Figure 1A and 1B). At the 24 hr time-point following radiocontrast exposure, the clonidine 10μg/kg bolus cohort (Cr=1.0±0.1, n=15, p=0.047), the clonidine 10μg/kg bolus + 5μg/kg/h infusion cohort (Cr=0.7±0.2, n=10, p=0.010), and the clonidine 10μg/kg bolus + 10μg/kg/h infusion cohort (Cr=0.8±0.1, n=10, p=0.013) revealed lower plasma Cr concentrations than saline treated mice, indicating preserved glomerular filtration and less nephropathy.

Dex treatment (3μg/kg bolus + 4μg/kg/h infusion cohort (Cr=0.8±0.1, n=10, p=0.008) also reduced the subsequent rise in plasma creatinine observed following iohexol injection. Mice administered dex bolus only (3μg/kg, no infusion) were not protected from radiocontrast-induced nephropathy (Cr = 1.5±0.4, n=8, p=0.871).

After 16h water deprivation and prostanoid and nitric oxide synthesis inhibition, clonidine (10μg/kg bolus), dex (3μg/kg bolus + 4μg/kg/h infusion), or yohimbine (0.1mg/kg bolus) administration alone (clonidine, dex, or yohimbine sham), without
iohexol injection, did not alter plasma creatinine concentrations \( (Cr=0.4\pm0.1, n=4, \) clonidine sham; \( Cr=0.5\pm0.1, n=4, \) dex sham; \( Cr=0.4\pm0.1, n=4, \) yohimbine sham).

Smaller doses of clonidine and dex, although insignificant, revealed dose response tendencies towards reductions in creatinine rise after iohexol injection compared to saline controls (clonidine 5\( \mu \)g/kg bolus cohort \( (Cr=1.1\pm0.2, n=7, p=0.226) \), clonidine 5\( \mu \)g/kg bolus + 5\( \mu \)g/kg/h infusion cohort \( (Cr=0.9\pm0.2, n=10, p=0.100) \), and dex 3\( \mu \)g/kg bolus + 2\( \mu \)g/kg/h infusion cohort \( (Cr=1.0\pm0.2, n=8, p=0.109) \).

Yohimbine, a selective alpha-2 antagonist, abolished the protective effects observed in clonidine and dex treated animals. The significant reductions in plasma Cr observed in the clonidine 10\( \mu \)g/kg/h + 10\( \mu \)g/kg cohort and the dex 4\( \mu \)g/kg/h + 3\( \mu \)g/kg cohorts were reversed by the addition of yohimbine, 0.1mg/kg, injected at the time of mini-osmotic pump placement and 15 minutes prior to clonidine or dex bolus injection (\( Cr=1.5\pm0.4, n=6, p=0.031 \), clonidine 10\( \mu \)g/kg/h + 10\( \mu \)g/kg + yohimbine 0.1mg/kg x2 vs. clonidine 10\( \mu \)g/kg/h + 10\( \mu \)g/kg; \( Cr=1.5\pm0.2, n=10, p=0.002 \), dex 4\( \mu \)g/kg/h + 3\( \mu \)g/kg + yohimbine 0.1mg/kg x2 vs. dex 4\( \mu \)g/kg/h + 3\( \mu \)g/kg). Moreover, these plasma creatinine concentrations were indistinguishable from saline iohexol controls (\( Cr=1.5\pm0.4, n=6, p=0.959 \), clonidine 10\( \mu \)g/kg/h + 10\( \mu \)g/kg + yohimbine 0.1mg/kg x2; \( Cr=1.5\pm0.2, n=10, p=0.890 \), dex 4\( \mu \)g/kg/h + 3\( \mu \)g/kg + yohimbine 0.1mg/kg x2). Yohimbine did not produce nephropathy when injected without iohexol (\( Cr=0.38\pm0.1, n=4, p=1.000 \), yohimbine sham). Yohimbine alone also did not exacerbate RCN after iohexol injection (\( Cr=1.6\pm0.2, n=5, p=0.716 \)) compared to saline iohexol controls (Figure 1C).

We performed additional \textit{in vivo} studies to extend the course of radiocontrast nephropathy and examined plasma creatinine beyond 24 hrs. Six C57BL/6 mice were
subjected to RCN (iohexol injection after overnight water deprivation and indomethacin and L-NAME injection) after clonidine (10 µg/kg bolus and 5 µg/kg/hr infusion) or saline treatment. Our model of un-resuscitated RCN resulted in severe renal injury with high mortality as 3 out of 6 mice in the saline-vehicle group died before reaching day #2 and an additional mouse died before reaching day #3. In contrast, only 1 mouse died before reaching day #2 and the entire remaining 5 mouse survived to day #3 in the clonidine treated group. The plasma creatinine values at day #2 and #3 were 0.67±0.03 mg/dL (N=3) and 0.47±0.1 mg/dL (N=2) for the saline group, respectively. Clonidine treatment lead to plasma creatinine values of 0.58±0.09 mg/dL (N=5) and 0.45±0.07 mg/dL (N=5) at days #2 and #3, respectively.

Plasma concentrations of clonidine and dex

The plasma concentration of clonidine at the time of iohexol injection was 0.70±0.11 ng/ml (n=5). Dex plasma concentration at the time of iohexol injection was 0.51±0.07 ng/ml (n=5).

Histological findings

Histological evaluation of kidney sections from all mouse cohorts subjected to RCN revealed cortical proximal tubular necrosis, vacuolization and apoptosis. However, when compared to RCN saline control animals, clonidine (10µg/kg bolus + 10µg/kg/h infusion) and dex (3µg/kg bolus + 4µg/kg/h infusion) pretreated animals had significantly less proximal tubular necrosis (clonidine: 1.6±0.5 necrotic tubules/400x field, n=6, p<0.034; dex: 0.5±0.4, n=6; saline: 5.7±1.6, n=6, p<0.01). Alpha-2 agonist treated
animals also developed less cortical vacuolization (Figure 2). TUNEL staining revealed more apoptotic cells in renal cortices of iohexol exposed animals treated with saline than in animals treated with clonidine or dex (clonidine: 38±10 TUNEL positive cells/100x field, n=6, p<0.05 compared to saline; dex: 23±5, n=6 p<0.01 compared to saline; saline: 122±11, n=6, Figure 3).

Renal outer-medullary blood flow following radiocontrast exposure

Outer-medullary renal blood flow decreased rapidly and significantly in all animals following radiocontrast injection during the 120 minute experiment. However, clonidine (10μg/kg bolus) or dex (3μg/kg bolus) pretreatment preserved renal outer-medullary blood flow after radiocontrast exposure when compared to saline pretreatment. For the clonidine cohort, the linear mixed effect model revealed a lack of interaction between pretreatment and time, and overall effect analysis demonstrated significant differences between clonidine and saline renal blood flow profiles (p=0.020). For the dex cohort, the linear mixed effect model verified significant pretreatment and time interaction, suggesting unique renal blood flow profiles for dex and saline treated mice (p=0.004). Systemic blood pressure did not change significantly throughout the 90 minutes following iohexol injection in saline, clonidine, or dex cohorts (Figure 4).

Radiocontrast exposure and direct renal tubular toxicity in vitro

Isolated HK-2 proximal tubule cells experienced dose-dependent reductions in viability following 16 hour exposure to 50, 100, and 150 mg iodine/ml iohexol (91 ±9% viability, n=12, at 50mg/mL iodine iohexol exposure; 52 ±7%, n=12, at 100mg/mL...
iodine iohexol exposure; 24 ±7%, n=12, at 150mg/mL iodine iohexol exposure). Alpha-2 adrenergic agonist pretreatment failed to decrease the cytotoxicity secondary to iohexol exposure in HK-2 cells when compared to saline pretreatment (data not shown).

Iohexol also caused dose-dependent reductions in viability following 16 hour exposure to 50, 100, and 150 mg iodine/ml iohexol (71 ±3% viability, n=6, at 50mg/mL iodine iohexol exposure; 53±3%, n=6, at 100mg/mL iodine iohexol exposure; 8±2%, n=6, at 150mg/mL iodine iohexol exposure) in RPMEC. Either of the alpha-2 adrenergic agonist pretreatments again failed to decrease the cytotoxicity secondary to iohexol exposure in vascular endothelial cells, when compared to saline pretreatment (data not shown).
Discussion

We have revealed the renal protective effects of alpha-2 adrenergic receptor activation during radiocontrast exposure in mice. Administration of clonidine or dexmedetomidine, both alpha-2 agonists, reduced the rise in serum creatinine observed 24 hrs after iohexol injection. Yohimbine, an alpha-2 receptor antagonist, inhibited the protection observed in the alpha-2 agonist treated animals. Histological analysis of the protected alpha-2 agonist treated animals’ kidneys revealed decreased tubular necrosis, cellular apoptosis, and intracellular vacuolization as compared to saline treated controls. Renal medullary blood flow assessment revealed a preservation of blood flow following iohexol injection in alpha-2 agonist treated animals. Clonidine or dexmedetomidine pretreatment did not decrease the direct cytotoxicity of iohexol to cultured HK-2 cells or vascular endothelial cells *in vitro*.

The pathogenesis of RCN remains a topic of active research as the clinical diagnosis of RCN carries heavy clinical significance. Evidence from basic research supports several mechanisms including intense intra-renal arteriolar vasoconstriction with consequent medullary hypoxia, direct cellular toxicity, and stress-mediated oxidative renal cell injury (8, 31). A critical decrease in cortico-medullary oxygen supply vs. demand characterizes clinically observed RCN.

Adrenergic receptors mediate numerous biological functions including regulation of blood flow in all organ systems, including the kidney. Alpha-2 adrenergic receptors are primarily associated with presynaptic neurons, where their autocrine behavior reduces neuronal release of norepinephrine and, consequently, regulates autonomic sympathetic tone (32). Reductions in regional vascular resistance, mediated by alpha-2 adrenergic
agonism, may maintain renal medullary blood flow during radiocontrast exposure. Decreases in renal outer-medullary blood flow were observed in all animal cohorts exposed to radiocontrast. However, a significant reduction in this perfusion decrement was observed in alpha-2 agonist treated animals. This preservation of outer-medullary perfusion may explain the improved markers of renal physiology, namely lower serum creatinine, and decreased markers of tissue injury, specifically less tubular necrosis, intracellular vacuolization, and apoptosis, 24 hours after radiocontrast injection.

With our murine model of RCN, we utilized a clinically applicable dose of radiocontrast (1.5g iodine/kg iohexol). Although the volume of radiocontrast administered in clinical practice depends on the requirements of each exam or procedure, coronary diagnostic angiograms often require 0.3g iodine/kg, CT scans, 0.7 g iodine/kg, peripheral vascular interventions, 0.5g iodine/kg, and interventional coronary imaging, 1.6g iodine/kg. Our choice of radiocontrast agent, iohexol, also reflects common clinical practice. It is a low-osmolar nonionic dimeric iodinated contrast media. We also chose in vitro concentrations of iohexol (50-150 mg iodine/ml) to mimic the renal tubular concentration achieved in clinical practice. For example, the routine injection of radiocontrast media results in plasma concentrations of ~10-20 mg iodine/ml (9). After ~60-80% of the water and solute content of the glomerular filtrate is reabsorbed into the blood stream from the proximal renal tubule, local cellular iodine concentrations substantially rise, leading to proximal tubule iodine concentrations similar to those employed in our study. In rats, injection of 1.6g iodine/kg iohexol results in urinary iodine concentrations of 125 to 200 mg iodine/ml (4).
We chose clonidine or dexmedetomidine to activate alpha-2 adrenergic receptors. Both of these drugs are commonly used clinically and have proven safety profiles. Direct measurement of systemic blood pressure in mice demonstrated that the doses of clonidine and dexmedetomidine we studied do not lead to hypotension. Therefore, protective effects of clonidine and dexmedetomidine on renal cortico-medullary blood flow cannot be due to the changes in systemic blood pressure.

The plasma level measurements of clonidine (0.7 ng/ml) and dexmedetomidine (0.51 ng/ml) demonstrated that the observed preservation of medullary renal blood flow, maintenance of renal function, and reduced markers of tissue injury were achieved at concentrations commonly found in humans prescribed clonidine and dexmedetomidine. Specifically, during the management of hypertension, effective clonidine therapy is commonly achieved at steady state plasma concentrations of 0.8 ng/ml (19). Dexmedetomidine’s effects are achieved with a plasma concentration of 0.81 ng/ml (5, 19).

Differences in pharmacokinetics likely account for the discrepancy between the clonidine bolus only group, which showed both a significant reduction in plasma creatinine rise after iohexol exposure and preserved renal medullary blood flow, and the dexmedetomidine bolus only group, which showed no reduction in creatinine rise but preserved renal medullary blood flow (Figures 1A, 1B, 4). Dexmedetomidine’s relatively short elimination half-life of two hours (28) renders a dexmedetomidine bolus dose eliminated, while radiocontrast exposure persists throughout the 24-hour nephropathy experiment. Clonidine’s elimination half-life of twelve-sixteen hours (19) accounts for extended alpha-2 agonism and consequently, a reduced rise in plasma creatinine, 24-
hours after exposure to radiocontrast. A continuous infusion of clonidine is not required. Dexmedetomidine mediated renal protection requires a continuous infusion throughout the 24-hour nephropathy experiment. Since the 120-minute renal medullary blood flow experiments complete prior to elimination of either a clonidine or dexmedetomidine bolus, drug infusion remains unnecessary during these blood flow studies.

We did not observe protection from the cytotoxic effects of iodinated radiocontrast administration in alpha-2 agonist pretreated proximal tubule (HK-2) cells or vascular endothelial cells in vitro. Both of these cell types contain alpha-2 adrenoreceptors (10, 14). Tubular toxicity, a consistent finding in isolated proximal tubule cells (8), was observed in all groups of HK-2 cells exposed to radiocontrast. This finding remains specific to iodinated radiocontrast exposure. Equivalent changes in cell media osmolality or volume, as achieved with mannitol, have not led to similar cytotoxicity (16). Following exposure to escalating concentrations of radiocontrast media, the viability of clonidine-, dexmedetomidine-, and saline-pretreated cells was equivalent. Although alpha-2 agonists failed to protect isolated proximal tubule cells or vascular endothelial cells from direct toxicity, radiocontrast mediated cytotoxicity is exacerbated by reperfusion injury (33). Consequently, preserved regional blood flow may limit cytotoxicity in vivo, even though cell culture experiments failed to show benefit to radiocontrast exposure, in the presence of alpha-2 agonists.

To better investigate the mechanisms and possible therapies for RCN, we developed a murine model of RCN. One of the limitations of this study is that our model utilizes young and healthy mice. These mice may not comprehensively mimic the infirmed patients that typically develop clinical RCN. Patients at high risk for developing
RCN frequently are intravascularly dehydrated and have exhausted the cellular mechanisms that maintain medullary oxygen delivery (24). Therefore in our murine model, we withheld water for 16 hrs prior to iohexol injection, and we inhibited prostanoid and nitric oxide synthesis. With these interventions, we were able to create a reliable and consistent model of RCN, a model that has been previously described in mice and rats (6, 12, 16).

We attempted to extend the course of RCN in this study to examine plasma creatinine beyond 24 hrs. However, our model of un-resuscitated RCN resulted in severe renal injury leading to high mortality (50%) in less than 48 hrs for the saline-treated mice. Although, the plasma creatinine values were similar between saline-treated and clonidine-treated mice subjected to RCN at day #2, we believe that the saline-treated mice that survived did so because they had reduced renal injury compared to the mice that died. These surviving mice, as well as their plasma creatinine values, reflect a selection bias. One of the limitations of our study is that due to the severe nature of our RCN model, we were unable to conclusively examine whether alpha-2 agonist-mediated renal protection persists beyond 24 hrs.

In summary, we have demonstrated that clonidine and dexmedetomidine attenuate the reduction in renal blood flow following radiocontrast injection and attenuate the development of subsequent nephropathy. Developing renal protective therapies remains vital to decreasing the morbidity and mortality associated with radiocontrast exposure. Reductions in renal function following radiocontrast exposure may be permanent, and 40% of patients meeting the diagnostic criteria for RCN may require hemodialysis (21). Whether alpha-2 agonists benefit humans subjected to the diagnostic and therapeutic
procedures that utilize iodinated radiocontrast media remains to be determined. Furthermore, whether alpha-2 adrenergic agonist mediated renal benefits surpass those of intravascular hydration alone warrants investigation.
**Disclosures:** None of the authors have financial ties to commercial companies.
Legends

Figure 1. (A) Plasma creatinine values of saline sham, clonidine (clon; 10µg/kg/h) sham, saline radiocontrast nephropathy (RCN), and clon (5µg/kg bolus; 10µg/kg bolus; 5µg/kg bolus + 5µg/kg/h infusion; 10µg/kg bolus + 5µg/kg/h infusion; 10µg/kg bolus + 10µg/kg/h infusion) RCN mice. RCN mice were injected with iohexol 24 hrs prior. (B) Plasma creatinine values of saline sham, dexmedetomidine (dex; 4µg/kg/h) sham, saline (100µl) RCN, and dex (3µg/kg bolus; 3µg/kg bolus + 2µg/kg/h infusion; 3µg/kg bolus + 4µg/kg/h infusion) RCN mice. RCN mice were injected with iohexol 24 hrs prior. (C) Plasma creatinine values of saline sham, yohimbine (yo) sham, saline RCN, clon (10µg/kg bolus + 10µg/kg/h infusion) + yo (0.1mg/kg x2) RCN, dex (4µg/kg bolus + 3µg/kg/h infusion) + yo (0.1mg/kg x2) RCN mice. RCN mice were injected with iohexol 24 hrs prior. *p<0.05 vs. saline RCN. #p<0.05 vs. saline sham. Error bar represents 1 SEM.

Figure 2. Representative light microscopy photographs of renal cortices from saline sham, saline radiocontrast nephropathy (RCN), clonidine (10µg/kg bolus + 10µg/kg/h infusion) RCN, and dex (3µg/kg bolus + 4µg/kg/h infusion) RCN mice (haematoxylin and eosin staining, magnification x 200). Saline-treated mice subjected to RCN show increased vacuolization of renal cortices (representative of 6 experiments). Clonidine sham and dex sham photos are not shown but they were indistinguishable from saline sham photos.
Figure 3. Representative fluorescent microscopy of renal juxtaglomerular junction of sham, saline radiocontrast nephropathy (RCN), clonidine (10µg/kg bolus + 10µg/kg/h infusion) RCN, and dex (4µg/kg bolus + 3µg/kg/h infusion) RCN mice (TUNEL staining, magnification x 200). Saline-treated mice subjected to RCN show increased apoptosis of renal cortices (representative of 6 experiments).

Figure 4. (A) Outer medullary renal blood flow and (B) systolic systemic blood pressure during initiation of radiocontrast nephropathy injury and for 90 minutes following iohexol injection in saline, clonidine (10µg/kg), and dex (3µg/kg) pretreated mice. *p<0.05: clonidine (10µg/kg bolus) and dex (3µg/kg bolus) vs. saline.
References


Figure 1A

Plasma Cr (mg/dl)

- Saline Sham
- Saline RCN
- Clon 10ug/kg/h Sham
- Clon 5ug/kg/h Sham RCN
- Clon 10ug/kg/h + 5ug/kg/h RCN
- Clon 5ug/kg/h + 10ug/kg/h RCN
- Clon 10ug/kg/h + 10ug/kg/h RCN

Legend:
- # indicates significant difference compared to Saline Sham
- * indicates significant difference compared to Clon 10ug/kg/h Sham
Figure 1B

Plasma Cr (mg/dl)

- Saline Sham
- Dex 4ug/kg/h Sham
- Saline RCN
- Dex 3ug/kg RCN
- Dex 2ug/kg/h+3ug/kg RCN
- Dex 4ug/kg/h+3ug/kg RCN

*#
Figure 1C

![Figure 1C](image)

**Plasma Cr (mg/dL)**

- Saline Sham
- Yo 0.1mg/kg Sham
- Saline RCN
- Yo 0.1mg/kg RCN
- Clon + Yo 0.1mg/kg RCN
- Dex + Yo 0.1mg/kg RCN

# denotes significant difference compared to the control group.
Figure 3

- Sham
- Saline RCN
- Clonidine RCN
- Dexmedetomidine RCN
Figure 4

A) Systolic Blood Pressure (mmHg)

B) Corticomedullary Blood Flow (% of Baseline)