Preclinical Studies of a Kidney Safe Iodinated Contrast Agent

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ABSTRACT

BACKGROUND AND PURPOSE: Contrast-induced acute kidney injury (CI-AKI) is a serious complication of the use of iodinated contrast agents. This problem is particularly acute in interventional neurology and interventional cardiology, probably due to the intra-arterial route of injection, high contrast volumes, and preexisting risk factors of these patients. In an attempt to develop a contrast agent that is less damaging to the kidneys, we have studied the effects of adding a small amount of the substituted cyclodextrin, sulfobutyl-ether-β-cyclodextrin (SBECD), to iohexol in rodent models of renal toxicity.

METHODS: Renally compromised mice and rats were injected with iohexol and iohexol-SBECD via the tail vein. The renal pathology, creatinine clearance, and survival benefits of iohexol-SBECD were studied. The safety of direct intra-arterial injection of the iohexol-SBECD formulation was studied in a dog heart model system. Mechanism of action studies in cell culture model using a human kidney cell line was performed using flow cytometry.

RESULTS: Nephrotoxicity was significantly reduced using iohexol-SBECD compared to iohexol alone, at mole ratios of iohexol:SBECD of 1:0.025. SBECD increased survival from 50% to 88% in a rat survival study. In the dog heart model, iohexol-SBECD was safe. Cell culture studies suggest that SBECD interferes with the early stages of contrast-induced apoptosis in a human renal cell line.

CONCLUSION: We have shown that the addition of a small amount of SBECD (one molecule of SBECD per 40 iohexol molecules) significantly protects rodent kidneys from CI-AKI. Further development of this new formulation of iodinated contrast is warranted.

Keywords: Contrast renal toxicity, kidney protection from contrast, iodinated contrast safety, safe contrast for interventional procedures, acute kidney injury.

Acceptance: Received March 11, 2016. Accepted for publication March 30, 2016.

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Acknowledgments and Disclosures: Vernon D. Rowe is CEO and Founder of Verrow Pharmaceuticals, Inc., and is author of patent US 8,277,779 B2 on iohexol:SBECD. MR Gralinksi is CEO and Founder of CorDynamics, Inc.
No other financial disclosures.

J Neuroimaging 2016;26:511-518.
DOI: 10.1111/jon.12356

Introduction

Contrast-induced acute kidney injury (CI-AKI) or contrast-induced nephropathy (CIN) is well established to be a significant side effect of the use of intravenous (IV) and intra-arterial administration of iodinated contrast agents.[1] This problem is particularly acute in interventional neurology and interventional cardiology, because intra-arterial injections are used, large high concentration contrast volumes are needed, and many patients who have to be studied have other risk factors for CI-AKI.

It has been shown in many studies that CI-AKI is associated with greater mortality and morbidity after interventional cardiology procedures.[2,3] Acute renal failure (ARF) was associated with poor outcomes in intracerebral hemorrhage patients, including those who had a cerebral angiography procedure.[4] Fewer studies of CI-AKI have been done in interventional neurology, but the volumes of contrast used are similar to those used in interventional cardiology.[5,6]

The incidence of CI-AKI varies among patients undergoing the many different types of interventional procedures, and can range up to 25% to 50% for high-risk patients.[1,3] CI-AKI has been estimated to be the third leading cause of hospital-acquired ARF and is responsible for 12% of ARF in hospitals.[7] Clearly, the problem of CI-AKI represents a serious medical complication that affects a large number of patients.

CI-AKI has been recognized for many years and many attempts to solve it have been undertaken.[1,8,9] However, in spite of all of these efforts, and the failure of many clinical trials, the current standard of care is relegated to pretest IV hydration, and minimization of the volume of iodinated contrast used.

Substituted cyclodextrins (CDs) have long been used as inert excipients in many drug preparations. In particular, sulfobutyl-ether-β-cyclodextrin (SBECD, trade name Captisol, Ligand) is used to solubilize otherwise insoluble drugs for IV injection. For example, FDA approved drugs that include SBECD in their formations include Nexterone (amiodarone HCL from Baxter), VFend (voriconazole from Pfizer), Geodon (ziprasidone HCL from Pfizer), Cerenia (maropitant citrate from Pfizer), Abilify (aripiprasol from Bristol-Myers Squibb), and Kyprolis (carfizomib from Onyx). In all of these formulations, and additional

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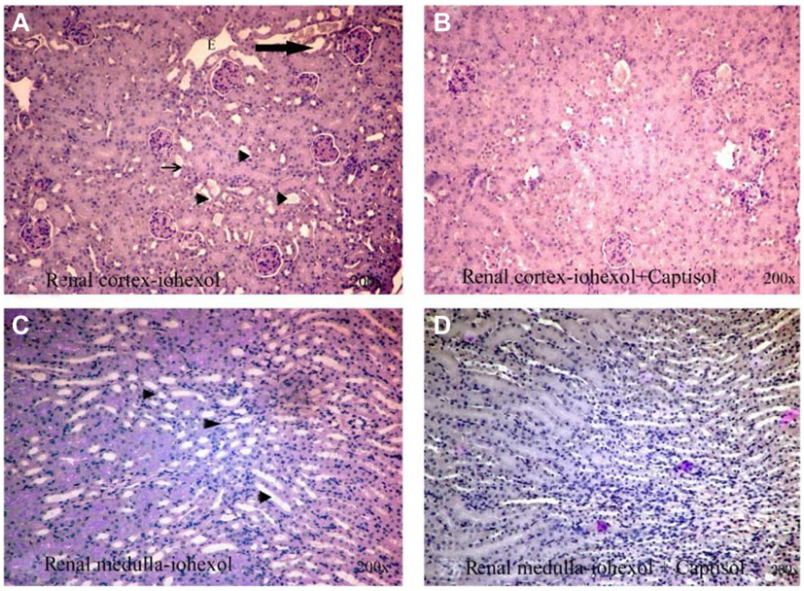

Fig 1. Light microscopy of renal tissue of mouse (H&E, PAS, 200×). Iohexol-treated kidneys indicate pathological changes in the renal cortex (A) and medulla (C) such as tubular vacuolation, tubular dilatation (big arrow), cast formation (thin arrow), loss of brush border (arrow heads), and focal edema (E). Concurrent SBECD (sulfobutyl-ether β cyclodextrin) administration at mole ratio 1:0.025 iohexol:SBECD significantly attenuated the morphological changes in both cortex (B) and medulla (D).

drug formulations in development, SBECD is considered an inert excipient.[10]

In an attempt to reduce the renal toxicity of IV methotrexate (MTX), when given intravenously to patients with multiple sclerosis (MS),[11] we undertook a study of the use of small amounts of SBECD reformulated with MTX in an animal model of MS. We found no precipitation of MTX in treated mice kidneys, but did find marked kidney tubule damage that was mitigated by reformulating with SBECD.[12] Following up on the results with high-dose IV MTX, we found similar kidney protection with iodinated contrast agents, cisplatin, doxorubicin, and gentamycin,[12,13] though the mole ratios of cyclodextrin to active agent varied with each agent.

The present report presents preclinical data for a new formulation of the most widely used iodinated contrast agent iohexol (Omnipaque from GE Healthcare) with SBECD.

Materials and Methods

Contrast reformulation: Omipaque 300 (GE Healthcare), Isovue-M 200 (Bracco Diagnostics), or Visipaque 320 (GE Healthcare) were diluted 1:1 with PBS, and then solid SBECD (Captisol, Ligand Pharmaceuticals) was added and dissolved in various mole ratios. The iohexol doses in rodents were 1.5 g iodine/kg (3.23 g iohexol/kg).

Iohexol (dog studies): Aqueous formulations were prepared containing 350 mg iodine/mL iohexol (Hovione FarmaCiencia SA), 0 or 50 mg/mL SBECD, 0.105 mg/mL edetate calcium disodium hydrate, and 1.21 mg/mL TRIS buffer (pH 6.8-7.7) in purified water. Preparations contained either 0 or 50 mg/mL SBECD at a mole ratio of 1:0.025 iohexol:SBECD.

Methods

Rodent Pathology Model

The rodent model of contrast media kidney damage of Agmon was used.[14] Renal compromise (RC) was induced in female C57BL/6 mice (8-10 weeks) and Sprague Dawley male rats (9-11 weeks) with a 10 mg/kg intraperitoneal (IP) injection of L-NAME (N-nitro-L-arginine methyl ester) followed in 10 minutes with 10 mg/kg indomethacin. The test formulations were dosed 20 minutes later as single 10 mL/kg injections into the tail vein at 1.5 g iodine/kg.[14]

Animals were sacrificed with rapid inhalation anesthesia at 24 or 48 hours postdosing and the kidneys removed and stored in buffered formalin. They were mounted in paraffin blocks, cut into 5 μM sections, and stained with hemolysin and eosin (H&E) and periodic acid Schiff (PAS). The sections were examined by light microscopy and scored for pathology in a blinded fashion.

Blood samples for creatinine determination were taken predose (IV) and at sacrifice (cardiac puncture). The plasma was isolated and stored at −70°C until assayed. Creatinine was measured calorimetrically with QuantiChrom Creatinine Assay Kit (BioAssay Systems, Hayward, CA, USA).

Pathology Evaluation

Kidney sections were evaluated at 400× magnification. Five randomly selected fields in each section were assessed for the occurrence of: dilated tubules, edema/mononuclear infiltration, loss of brush border, vacuoles, tubular casts, and tubular degradation, and scored in a blinded manner on a scale of 0–4. A total of four sections were analyzed per kidney. The 20 assessments for each parameter were averaged and reported as an average score per field. Total pathology score, a summation of the average scores for the six parameters, is used in some figures for efficiency of presentation. All error bars are SEM (standard error of the mean).

Rodent Survival Model

Male Sprague Dawley rats (9-11 weeks, 8/group) received IP injections of L-NAME and indomethacin as described above, followed by IV placebo, iohexol or iohexol-SBECD

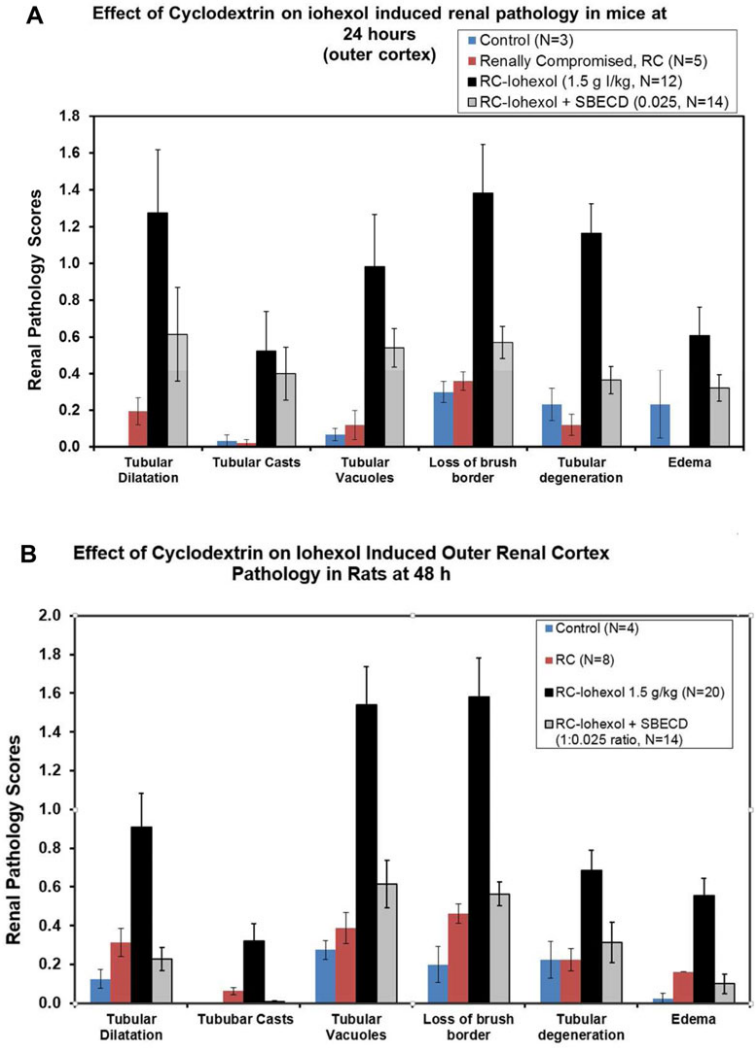

Fig 2. (A) Effect of adding SBECD (sulfobutyl-either β cyclodextrin) to iohexol on mouse outer renal cortex pathology measurements for six parameters at a mole ratio of iohexol:SBECD of 1:0.025. (B) Effect of adding SBECD to iohexol on rat outer renal cortex pathology measurements for six parameters at a mole ratio of iohexol:SBECD of 1:0.025.

(1:0.025 mole ratio) at 2.5 g Iodine(I)/kg. Their survival was then monitored for 14 days.

HK-2 Cell Culture

HK-2, an immortalized proximal tubular cell line derived from normal adult human kidney, was obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). HK-2 cells were maintained in keratinocyte serum-free medium (K-SFM) supplemented with 0.05 mg/mL bovine pituitary extract (BPE) and 5 ng/mL human recombinant epidermal growth factor (EGF). All components of the growth medium were purchased from Invitrogen (Life Technologies, Carlsbad, CA). These cells were routinely cultured at 37°C in a humidified incubator with 95% air to 5% CO_2 and medium was replaced every 3–4 days.

HK-2 Cell Apoptosis and Cytotoxicity Assays

HK-2 (15,000 cells/cm^2) cells were seeded in six-well plates in growth medium at 37°C in a humidified atmosphere containing 5% CO_2 for 24 hours. Cell apoptosis was measured using a CellEvent™ Caspase-3/7 Green Flow Cytometry Assay Kit and Pacific Blue™ Annexin V/SYTOX® AADvanced™ Apoptosis Kit, for flow cytometry (Life Technologies). Iohexol was diluted 1:1 in media and then solid SBECD was added and dissolved in various mole ratios: media only (0:0), 150 mgI/mL Iohexol only (1:0), 150 mgI/mL iohexol:SBCD mole ratio (1:0.025, 1:0.0125, 1:0.05, 1:0.10), and 20 mg/mL SBECD only (0:0.025). HK-2 cells were exposed to treatments for 1 hour. The cells were trypsinized and washed with phosphate buffered saline (PBS) (pH 7.4; Invitrogen), and incubated in binding buffer containing SYTOX Green, Caspase-3/7 and/or Annexin V. Flow cytometry analysis was performed using BD FACS DIVA (Becton Dickinson). For each measurement, 10,000 events were counted.

Instrumented Dog Cardiovascular Model

Male Beagle dogs were anesthetized with propofol, intubated, and placed on isoflurane gas anesthesia. Morphine (0.5 mg/kg) was used for pain management in this open chest procedure. A Swan-Ganz catheter for measurement of right heart pressure was inserted into the jugular vein, advanced to the pulmonary artery "wedge" position. A solid-state high-fidelity

pressure catheter (Millar) for left ventricular pressure and aortic pressure was inserted into the carotid artery. Both were secured with suture.

The surface lead ECG was recorded continuously via electrodes placed on the right arm, left leg, and chest of the dog. ECGs were continuously recorded throughout the experiment, reporting PR, QRS, QT, QTc (VdW), and heart rate. Monophasic action potential was recorded (when possible) via left ventricular epicardial probe.

Each formulation (iohexol or iohexol-SBECD) was bolused into the left main coronary artery as five doses of 4 mL each, administered at ~1 mL/second with 10 seconds between doses. Thirty minutes after the last dose, the procedure was repeated with the second formulation. The overall process was repeated in two additional animals.

Results

Kidney Pathology

A single dose of iohexol caused significant pathological changes in the kidneys of the RC rodents. The photomicrograph in Figure 1 illustrates the typical pathology seen at 24 hours in RC mice that received a single 1.5 g iodine/kg dose of iohexol or iohexol:SBECD in a mole ratio of 1:0.025. Iohexol treated kidneys showed pathological changes in both the renal cortex (Fig 1A) and medulla (Fig 1C) including tubular vacuolation, tubular dilatation (big arrow), cast formation (thin arrow), loss of brush border (arrow heads), and focal edema (Fig 1E). The presence of one molecule of SBECD per 40 iohexol molecules in the formulation significantly attenuated the morphological changes in both cortex (Fig 1B) and medulla (Fig 1D).

The corresponding representative quantitative kidney pathology scores are presented in Figure 2(A) for the outer renal cortex in mice at 24 hours, and in Figure 2(B) for the outer renal cortex of rats at 48 hours, showing the effects on six parameters from the analysis of photomicrographs such as those shown in Figure 1. These charts show significant pathology caused by iohexol in all of the parameters, with the largest effects on tubular dilation, vacuolization, and loss of brush border.

With the addition of SBECD, a reduction occurs on all six parameters in both species and is particularly significant in the reduction of pathology in tubular dilation, vacuolization, and loss of brush border. The pathology scores for all six parameters can be added to give a total pathology score, which is used in further presentations of the data.

Dose Response

Figure 3 shows the reduction in total pathology scored by SBECD in mice for both the outer renal cortex and the deep cortex/outer medulla as a function of the mole ratio of iohexol to SBECD. As seen here, the dose response is optimal between mole ratios of iohexol:SBECD of 1:0.05 and 1:0.025. At the ratio of 1:0.0125, there is no significant protection (data not shown). At the higher mole ratios, the protection is also reduced, leading to a U-shaped curve, suggesting that the mole ratio is critical for nephroprotection. The further studies were carried out at a mole ratio of iohexol to cyclodextrin of 1:0.025 (which equals only one cyclodextrin molecule for every 40 molecules of iohexol).

Figure 4 shows a summary of results for rats at 48 hours and at 24 hours at the mole ratio of iohexol:SBECD of 1:0.025.

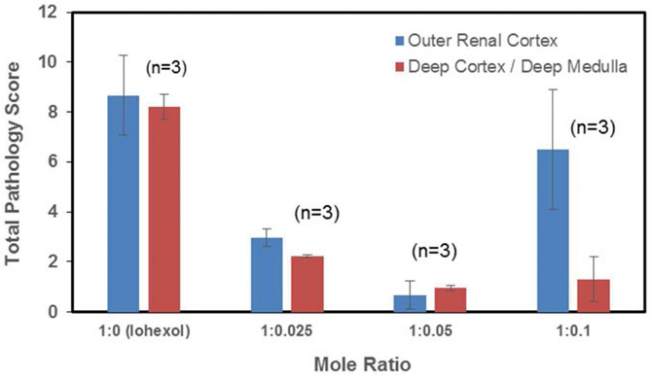

Fig 3. Dose effect of SBECD (sulfobutyl-either β cyclodextrin) addition to iohexol on mouse kidney pathology expressed as mole ratio of iohexol:SBECD. The total pathology scores were calculated by combining the six pathology parameters: tubular dilation, tubular casts, tubular vacuoles, loss of brush border, tubular degeneration, and edema.

This figure summarizes the results over many experiments and demonstrates the protection of both rat and mouse kidneys from renal pathology due to doses of iohexol that are comparable to those used in human interventional cardiological and neurological procedures.

Additional Iodinated Contrast Agents

Although iohexol (Omnipaque, GE Healthcare) is the most widely used iodinated contrast agent, other similar iodinated contrast agents are also used. Figures 5(A) and (B) show a comparison of the protective effects of SBECD on iohexol toxicity with those of iopamidol (Fig 5A) and iodixanol (Fig 5B). These charts demonstrate that these additional contrast agents cause renal pathology changes similar to that of iohexol, and that the addition of one molecule of SBECD per 40 molecules of contrast agent is sufficient to provide significant protection from these changes.

Serum Creatinine Changes

The effect of iohexol on kidney function was also measured by following serum creatinine in both rats and mice. Figure 6 shows that in mice, contrast administration resulted in an increase in serum creatinine, which was mitigated by the addition of SBECD to iohexol. Rats, which are known to be particularly resistant to functional renal damage, did not show a significant increase in creatinine with iohexol treatment. However, rats did show a significant functional benefit of SBECD in the survival study (Fig 7).

14-Day Survival Study

Figure 7 shows the results of a survival study of rats that were administered iohexol or iohexol plus SBECD and followed for 14 days. The nephrotoxicity of contrast agents can lead to mortality in rodents when larger doses are administered. Only half (four of eight) survived a single dose of 2.5 g I/kg iohexol as shown in Figure 7. The presence of SBECD at an iohexol:SBECD mole ratio of 1:0.025 reduced the nephrotoxicity and raised survival to 88% (seven of eight). The SBECD rat that died had clear symptoms of infection; thus, his death could not be attributed to the iohexol injection alone. These

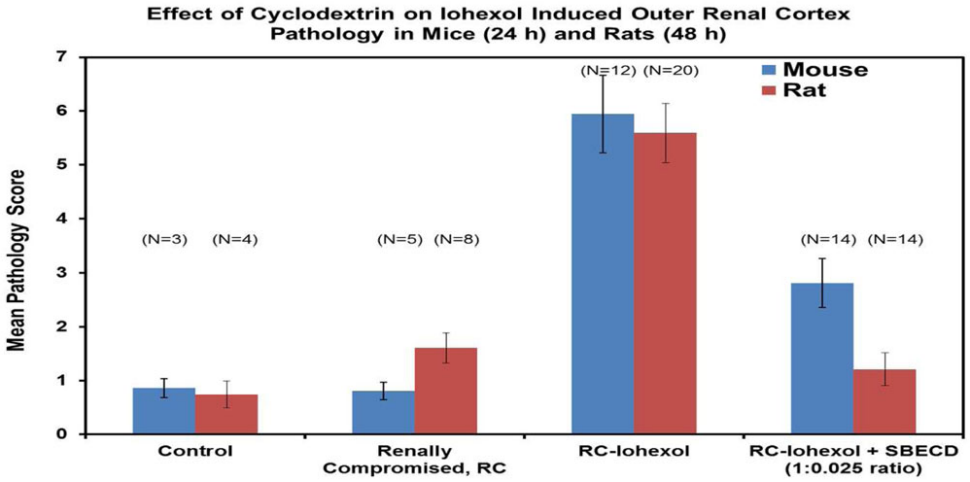

Fig 4. Summary of rat and mouse pathology data, total pathology scores calculated by combining pathology scores for tubular dilation, tubular casts, tubular vacuoles, loss of brush border, tubular degeneration, and edema.

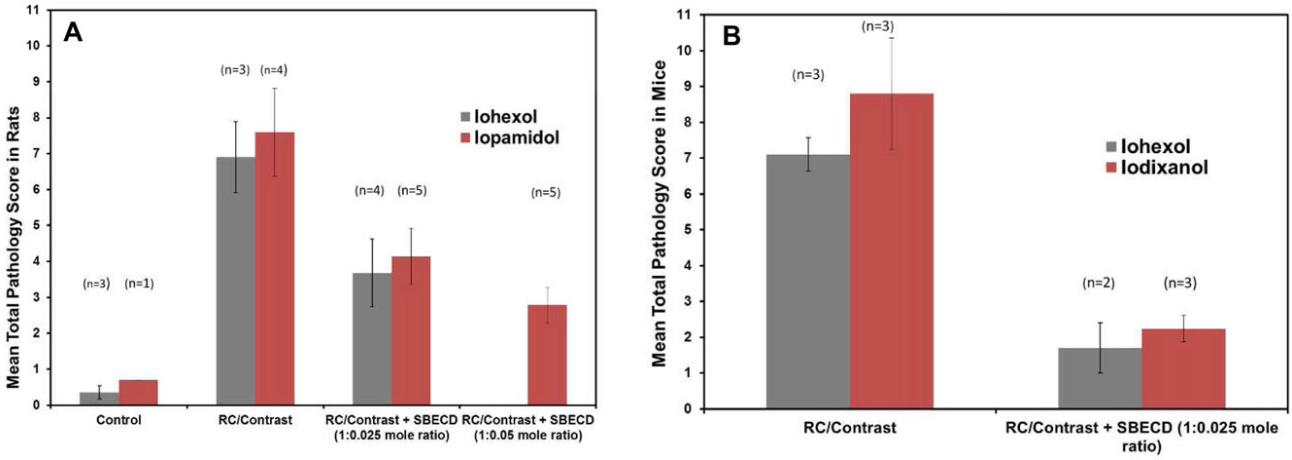

Fig 5. (A) Comparison of kidney protection by SBECD (sulfobutyl-either β cyclodextrin) for iopamidol and iohexol in rats at mole ratio 1:0.025, iohexol:SBECD or iopamidol:SBECD. (B) Comparison of kidney protection by SBECD for iodixanol and iohexol in mice at mole ratio of 1:0.025, iohexol:SBECD or iodixanol:SBECD.

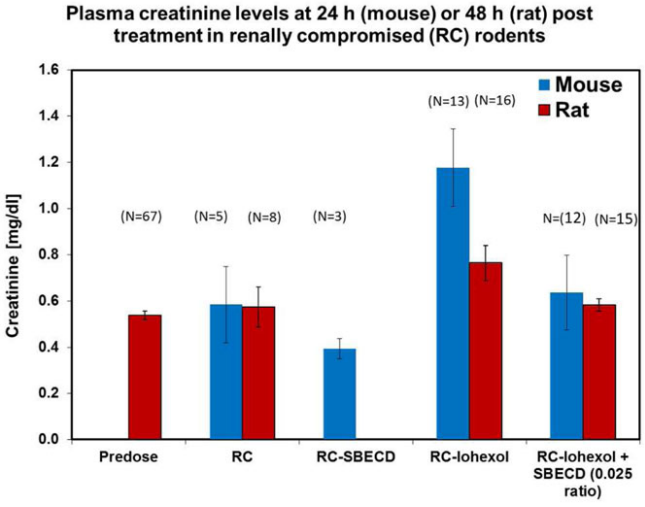

Fig 6. Serum creatinine levels at 24 h (mouse) or 48 h (rat) post treatment with iohexol or iohexol-SBECD (sulfobutyl-either β cyclodextrin) in renally compromised (RC) rodents at mole ratio 1:0.025.

data show that the addition of SBECD to iohexol increased the survival rate for 14 days from 50% to 88%.

Cell Culture Studies of Nephroprotective Mechanism

Figure 8 shows the results of cell culture experiments using flow cytometry to detect apoptosis in cultured HK-2 cells that had been incubated with iohexol and varying mole ratios of SBECD. Both Anexin V and Caspase 3/7 labeling showed the iohexol induction of apoptosis and protection by SBECD. It is notable that the dose response in this system is similar to that seen in the pathology studies, with a nonlinear U-shaped function and an optimum mole ratio of iohexol:SBECD around 1:0.025.

Instrumented Dog Heart Studies–Cardiac Safety

The instrumented dog heart model was used to evaluate the effect of SBECD on the tolerability of interarterial injection of contrast in a model that is routinely used for preclinical studies of contrast agents to be used for cardiovascular studies. The effect of iohexol formulated with SBECD (1:0.025 mole ratio) on the electrophysiology of the heart was compared to that of

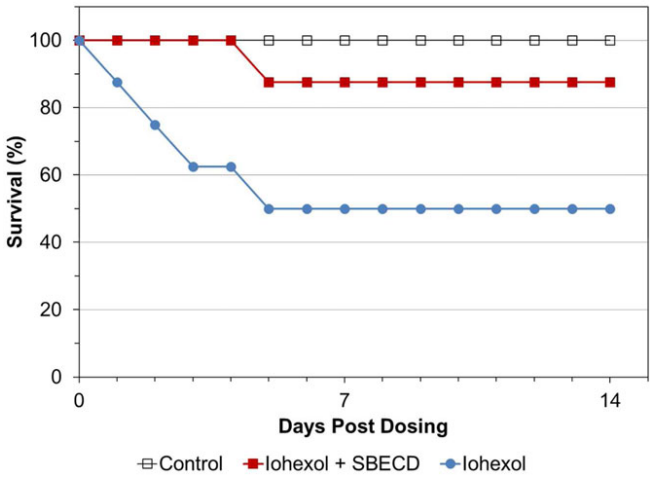

Fig 7. Survival of RC rats receiving single IV 2.5 g I/kg doses of iohexol or iohexol + SBECD (sulfobutyl-either β cyclodextrin) at mole ratio of 1:0.025 iohexol:SBECD. $N = 8$ in each group.

iohexol after direct injection into the left coronary artery of instrumented dogs.

There were no notable effects of intracoronary iohexol administration on most measured cardiovascular parameters. Figure 9 shows that both formulations of iohexol, with and without SBECD, had similar effects on the dog heart. Variables including LV contractility (Fig 9A) and QTc interval (Fig 9B) were notably, yet transiently, altered following both the iohexol and iohexol + SBECD regimens.

In addition to these transient quantitative changes, qualitative alterations in electrocardiographic morphology were observed for both formulations. These were generally concomitant with physical injection of the formulations into the coronary artery, and likely associated with brief myocardial ischemia from interruption of arterial flow. The changes consisted of QRS complex widening along with ST segment depression. Scattered premature ventricular contractions were also noted. Within 5 minutes after the end of each injection, electrocardiogram morphology had returned to normal for each formulation. The data are in agreement with the published literature on iohexol injections.[15]

Discussion

Our results show that the addition of a small amount of the substituted cyclodextrin SBECD to iodinated contrast agents significantly reduces kidney injury in rodents at contrast doses that are similar to those used clinically in interventional neurology and cardiology. The decrease in nephrotoxicity is particularly evident in the survival study, showing that the addition of SBECD had no adverse effects, but rather was protective. The dog heart studies show that the addition of SBECD to iohexol has no effect on the response of the heart to iohexol alone.

This discovery is a significant advance in efforts to find a way to mitigate the serious medical problem of CI-AKI in interventional neurology and cardiology, and all other uses of iodinated contrast agents. Although there have been attempts to alter the molecular structure of iodinated contrast, none of these changes in the structure of low ionic or isoionic iodinated contrast agents have made a significant impact on the incidence of CI-AKI.[8,9,16]

Substituted Cyclodextrin SBECD

The substituted cyclodextrin SBECD has been used in the development of several useful FDA-approved pharmaceutical agents, as detailed above, and is considered to be an inert excipient that solubilizes insoluble drugs by complexation.[10,17]

Our studies demonstrate that SBECD has a powerful biological activity that has not been recognized previously. The nephroprotective effect described here occurs at a mole ratio of iohexol to SBECD of 1:0.025, showing that complexation of the contrast by the SBECD cannot be the mechanism of action. Indeed, no significant association between iohexol and SBECD could be demonstrated (unpublished data). Thus, the mechanism of action involved in nephroprotection must involve an interaction of the SBECD with some element in the toxicity cascade of the kidneys.

Mechanism of Kidney Protection by SBECD

We have shown here that SBECD decreases the apoptosis caused by iohexol in cell culture. Many studies have shown that contrast-induced apoptosis proceeds through the mitochondrial pathway.[18–21] Involved in this pathway is the bcl-2 family of proteins, which are pro- and antiapoptotic proteins whose interactions and equilibrium regulate apoptosis. The proapoptotic Bax is primarily localized in the cytosol but is in dynamic equilibrium with a small proportion associated with the outer mitochondrial membrane in a healthy cell. When apoptosis is triggered, a conformational change occurs, changing the relative exposure of hydrophobic amino acids, and the terminal $\alpha 9$ transmembrane helix of Bax inserts into the outer mitochondrial membrane.[22] Associating into oligomers with other members of the bcl-2 family of proteins such as Bak, it forms large pores that cause increased membrane permeability and the release of cytochrome c into the cytosol, furthering the apoptotic cascade leading to cell death.[23–28] The involvement of Bax in contrast-induced apoptosis is supported by the finding of an increase in Bax in several tissues after exposure to iohexol.[19,20]

Substituted cyclodextrins are well known to bind hydrophobic amino acid side chains of proteins in their hydrophobic pocket. They shift the thermal denaturation of proteins to lower temperature,[29–32] and they have been used as "artificial chaperones" in renaturation of proteins in bacterial syntheses.[17,31,33] Since the initiation of the apoptotic cascade involves conformational changes including exposure of hydrophobic regions of Bax and Bac, it seems possible that SBECD binds with one or more of these proteins, interfering with the interactions of these proteins with the mitochondrial membrane or with each other, preventing the formation of the pores of the mitochondrial membrane and blocking the apoptotic cascade.

We have shown that the dose response in terms of the iohexol:SBECD mole ratio is not linear, but rather has a U-shaped function, indicating a hormetic character to the mechanism. A similar U-shaped curve for SBECD nephroprotection from methotrexate was found previously,[12] with its optimum mole ratio being 1:0.50, ie, much higher than found for iohexol, due to the greater nephrotoxicity of methotrexate. Hormetic mechanisms involve competing processes resulting in opposite effects in different dose ranges.[34,35] Cell signaling pathways are key examples of known hormetic systems, discussed by Calebrese.[34]

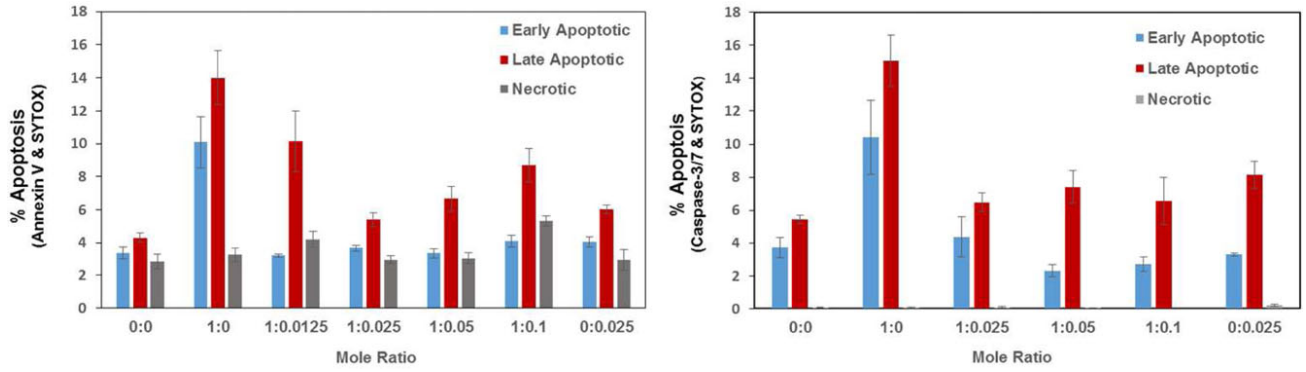

Fig 8. ***Annexin V, Caspase-3/7, and SYTOX Flow Cytometry.*** Protective effects of SBECD (sulfobutyl-either β cyclodextrin) against iohexol-induced apoptosis. HK-2 cells were exposed to treatment for 1 hour with indicated more ratio of SBECD:Iohexol. Mean percentage values were calculated by averaging the results from three independent experiments (***n*** = 2, ***n*** = 3, and ***n*** = 3, respectively). +/- SEM is shown.

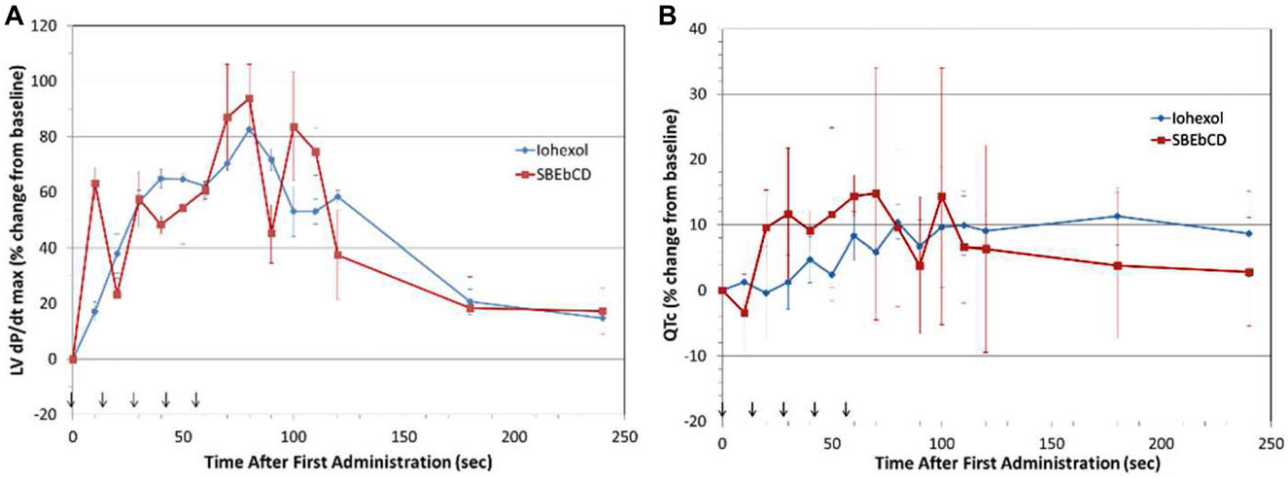

Fig 9. (A) Left ventricular contractility changes following bolus dosing into the left coronary artery of iohexol or iohexol:SBECD (sulfobutyl-either β cyclodextrin) at mole ratio of 1:0.025. (B) QTcV interval changes following bolus dosing into the left coronary artery.

In the case of the nephroprotective effect of SBECD, the competing mechanisms that may be involved are the interactions with the SBECD with more than one of the pro- and antiapoptotic cell-signaling proteins, such as those in the bcl 2 family. Our results suggest that the amount of the proapoptotic signal evoked by the insulting iohexol must be matched by a similar molar concentration of SBECD for maximum antiapoptotic effect; a greater relative amount of SBECD may interact with one of the ant-apoptotic signaling proteins, shifting the balance back toward apoptosis.

Conclusion

Our studies demonstrate for the first time that SBECD has significant biological activity. The discovery that this widely used "inert excipient" has such potent biological activity may have important implications for future drug development using this compound. This property allowed us to formulate iodinated contrast so that the new formulation became "kidney safe," for potential use in interventional cardiologic and interventional neurologic procedures, and for all of the uses of iodinated contrast agents. Further development of this contrast agent is warranted.

A mechanism of action of SBECD in nephroprotection is suggested, which involves interruption of the apoptotic cascade very early after the initial insult, by disrupting the hydrophobic protein–protein and protein–membrane interactions that lead to permeabilization of the mitochondrial membrane and the subsequent release of cytochrome c, thus blocking apoptosis at a very early stage. This activity could represent a novel mechanism that may allow the further development of other pharmacological agents that were previously limited in their use because of nephrotoxicity.

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