UNIVERSITÀ DEGLI STUDI DI NAPOLI

"FEDERICO II"

Scuola di Dottorato in Medicina Molecolare

Dottorato di Ricerca in Patologia e Fisiopatologia Molecolare

"Contrast agents and renal cell apoptosis"

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Dipartimento di Biologia e Patologia Cellulare e Molecolare "Luigi Califano"

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Dipartimento di Biologia e Patologia Cellulare e Molecolare "Luigi Califano"

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Tesi di Dottorato di Ricerca in Parologia e Fisiopatologia Molecolare
XXI ciclo

"*Contrast agents and renal cell apoptosis"*

Candidato: Dott.ssa Giulia Romano

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Introduction

Contrast-induced nephropathy (CIN) accounts for 10% of all causes of hospital-acquired renal failure, causes a prolonged in-hospital stay, and represents a powerful predictor of poor early and late outcome [1,2]. CIN is generally defined as an increase in serum creatinine concentration of >0.5 mg/dL (> 44 μmol/L) or 25% above baseline within 48 hours after contrast administration [3-7]. CIN is the acute deterioration of renal function after parenteral administration of radiocontrast media in the absence of other causes. CIN has gained increased attention in the clinical setting, particularly during cardiac intervention but also in many other radiological procedures in which iodinated contrast media are used [8].

There is at present good clinical evidence from well-controlled randomized studies that CIN is a common cause of acute renal dysfunction. Although the exact mechanisms of CIN are yet to be fully elucidated, several causes have been described [9-11].

Increased adenosine-, endothelin-, and free radical–induced vasoconstriction and reduced nitric oxide– and prostaglandin-induced vasodilatation have been observed [12].

These mechanisms cause ischemia in the deeper portion of the outer medulla, an area with high oxygen requirements and remote from the

vasa recta supplying the renal medulla with blood [13]. Contrast agents also have direct toxic effects on renal tubular cells, causing vacuolization, altered mitochondrial function, and apoptosis [7].

In Figure 1 is summarized the interplay of the various factors contributing to the pathogenesis of CIN, namely vasoconstriction, oxidative stress, and direct tubular toxicity leading to hypoxia of the outer medulla [14].

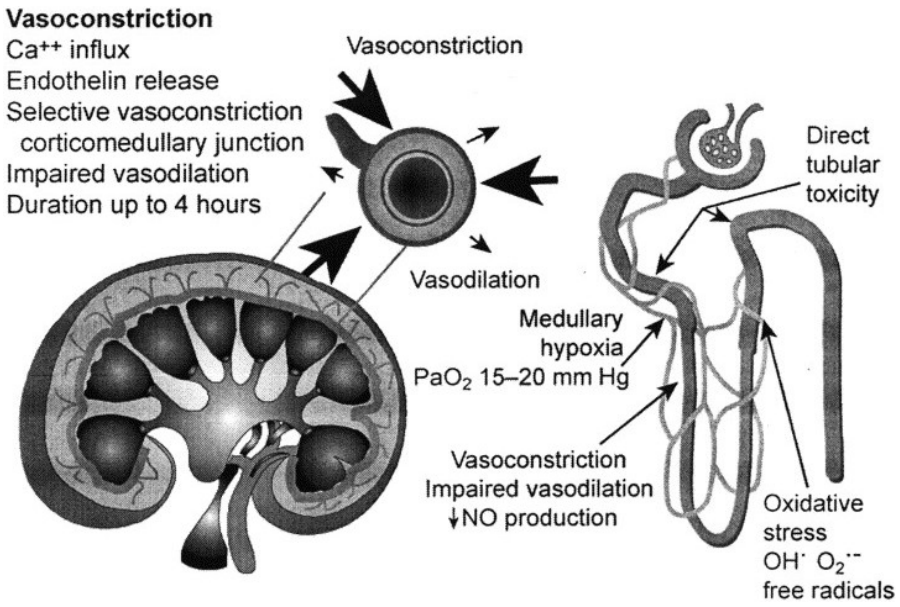


Figure 1. Overview of the factors involved in the pathogenesis of contrast-induced nephropathy. NO = nitric oxide; OH* = hydroxyl radical; $O2^{*-}$ = superoxide radical; PaO2 = arterial oxygen pressure.

Researchers in early in vitro studies found evidence for direct renal tubular cell toxic effects of contrast media [15,16]. In vitro experiments are a way to examine the cytotoxic effects of contrast media on renal cells because of the absence of confounding variables, which can be found in vivo (eg, hypoxia due to hemodynamic changes or other systemic mechanisms) [17-19].

Overview of renal physiology.

The kidneys are responsible for a number of important regulatory functions such as the maintenance of ion levels in the body, water retention/removal, waste excretion, blood pressure regulation and maintenance of proper blood acidity [20].

The kidney receives about 25% of cardiac output and the 80% of blood flow goes to the cortex and the remaining 20% portion to the medulla.

The cortex is the outer part of the kidney containing most of the nephrons. The medulla is the inner part of the kidney and contains the specialised nephrons in the juxta-medullary region, immediately next to the medulla. Nephrons are the functional unit of the kidney. Each kidney consists of about one million nephrons. The nephron is made up of a glomerulus and its tubule.

The tubule is made up of a number of sections, the proximal tubule, the medullary loop (loop of Henle), and the distal tubule which finally empties into the collecting duct [21] (fig.2)

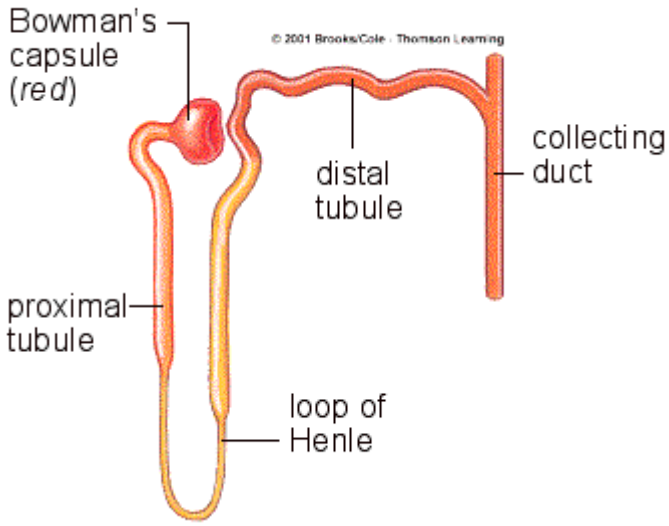


Fig.2 Schematic model of nephron

Filtration takes place through the semipermeable walls of the glomerular capillaries which are almost impermeable to proteins and large molecules. The filtrate is thus virtually free of protein and has no cellular elements. The glomerular filtrate is formed by squeezing fluid through the glomerular capillary bed. The driving hydrostatic pressure (head of pressure) is controlled by the afferent and efferent arterioles, and provided by arterial pressure. About 20% of renal plasma flow is filtered each minute (125 ml.min-1) [20].

The function of the renal tubule is to reabsorb selectively about 99% of the glomerular filtrate.

The Proximal Tubule reabsorbs 60% of all solute, which includes 100% of glucose and amino acids, 90% of bicarbonate and 80-90% of inorganic phosphate and water.

Reabsorption is by either active or passive transport. Active transport requires energy to move solute against an electrochemical or a concentration gradient. It is the main determinant of oxygen consumption by the kidney. Passive transport is where reabsorption occurs down an electrochemical, pressure or concentration gradient.

Most of the solute reabsorption is active, with water being freely permeable and therefore moving by osmosis. When the active reabsorbtion of solute from the tubule occurs, there is a fall in concentration and hence osmotic activity within the tubule. Water then moves because of osmotic forces to the area outside the tubule where the concentration of solutes is higher.

The Loop of Henle is the part of the tubule which dips or "loops" from the cortex into the medulla, (descending limb), and then returns to the cortex, (ascending limb). It is this part of the tubule where urine is concentrated if necessary. This is possible because of the high concentration of solute in the substance or interstitium of the medulla. This high concentration of solutes is maintained by the counter current multiplier. A counter current multiplier system is an arrangement by which the high medullary interstitial concentration of solute is maintained, giving the kidney the ability to concentrate urine. The loop of Henle is the counter current multiplier and the vasa recta is the counter current exchanger.

Actions of different parts of the loop of Henle:

A: The descending loop of Henle is relatively impermeable to solute but permeable to water so that water moves out by osmosis, and the fluid in the tubule becomes hypertonic.
B: The thin section of the ascending loop of Henle is virtually impermeable to water, but permeable to solute especially sodium and chloride ions. Thus sodium and chloride ions move out down the concentration gradient, the fluid within the tubule becomes firstly isotonic then hypotonic as more ions leave. Urea which was absorbed into the medullary interstium from the collecting duct, diffuses into the ascending limb. This keeps the urea within the interstitium of the medulla where it also has a role in concentrating urine.
C: The thick section of the ascending loop of Henle and early distal tubule are virtually impermeable to water. However sodium and chloride ions are actively transported out of the tubule, making the tubular fluid very hypotonic.

The Vasa Recta is a portion of the peritubular capillary system which enters the medulla where the solute concentration in the interstitium is high. It acts with the loop of Henle to concentrate the urine by a complex mechanism of counter current exchange. If the vasa recta did not exist, the high concentration of solutes in the medullary interstitium would be washed out.

Solutes diffuse out of the vessels conducting blood towards the cortex and into the vessels descending into the medulla while water does the

opposite, moving from the descending vessels to the ascending vessels. This system allows solutes to recirculate in the medulla and water, in effect, to bypass it.

In the renal medulla the O_2 tension is very small compared to the renal cortical portion (20-30 mmHg vs 50-60 mmHg). This effect is trigged to the lower O_2 contribution due to the reduction of blood flow and to the high oxygen need necessary to support the active tubular transport against concentration gradient. The direct O_2 permeation in vasa recta from venous to artery is a further cause of renal medulla hypoxia.
In normal kidney, the balance between the need of O_2 and blood flow is regulated by a balance of autocrine mediators with vasoconstriction activity (angiotensin, adenosine, ATP, endothelin, ADH) and vasodilatation activity (prostaglandins, NO, natriuretic peptide).

Oxygen-Free Radicals

A pathway that is proposed for develop of CIN is an increase in oxygen-free radicals or a decrease in antioxidant enzyme activity triggered by contrast medium administration. In some ways, this could be a sequence of the direct tubular cell toxicity pathway if the endogenous biochemical disturbances are simply the product of tubular cell damage rather than the primary cause of the resultant tubular cell damage. Free radicals are atoms or molecules that contain one or more unpaired electrons. In vivo, oxygen molecules are changed into water molecules after successive reduction reactions. Intermediate species are called “reactive oxygen species.” At high concentrations, free radicals have highly deleterious effects on all cellular constituents and cause oxidative stress and protein damage.

Free radicals react with (oxidize) various cellular components including DNA, proteins, lipids / fatty acids and advanced glycation-end products (e.g. carbonyls). These reactions between cellular components and free radicals lead to DNA damage, mitochondrial malfunction, cell membrane damage and eventually cell death (apoptosis).

Oxidative stress represents an excess of reactive oxygen species (ROS) in the tissues under consideration or in the whole body. This implies either an increased production of ROS, for example, by specific oxidase such as NADPH oxidase, xanthine/xanthine oxidase,

various arachidonic acid monooxygenases or the mitochondrial respiratory chain. Alternatively, it may derive from a failure to metabolize ROS. The major pathways for metabolism are superoxide dismutase (SOD), which is expressed as extracellular, intracellular, and mitochondrial isoforms that metabolize superoxide anion (O_2^-•) to H_2O_2. Peroxidases such as catalase and glutathione peroxidase (predominantly intracellular) further metabolize H_2O_2 to O_2 and water. However, such a definition is highly simplistic because of the other, biologically important, ROS such as hydroxyl anion (•OH) formed from H_2O_2, peroxynitrate ($ONOO^-$) formed principally by the interaction of nitric oxide (NO) and (O_2^-•) or •OH, or hypochlorous acid formed by myeloperoxidase. There are multiple other reaction products with other mediators. Important interactions occur between ROS and NO where (O_2^-•) not only reduces NO bioactivity by shortening its half-life but also generates highly reactive species such as $ONOO^-$ that are themselves implicated in oxidative and nitrosating reactions. No less problematic is the quantitative assessment of oxidative stress. At the level of the whole animal, use is often made of the appearance of oxidized end products of ROS metabolism. (Fig.3) These end-products include lipid peroxidation products such isoprostanes, which are formed predominantly non enzymically by the interaction of (O_2^-•) with arachidonate or malondialdehyde [22].

Furthermore the intense vasoconstriction and loss of autoregulatory capacity can contribute to additional renal injury through the release

of reactive oxygen species (eg, superoxide [OH].). Organ injury can occur when hypoperfusion of tissues generates reactive oxygen species that exceed the antioxidant reserve of the patient. The ability to accommodate oxidant injury decreases with age and is thought to contribute to the increased risk of CIN among older patients. Moreover, increased oxidative stress is present in chronic renal failure and in diabetes. It contributes to enhanced basal vascular tone and to impaired endothelium-dependent relaxation in chronic kidney disease. There are few data on the role of reactive oxygen species in the pathogenesis of CIN [23,24].

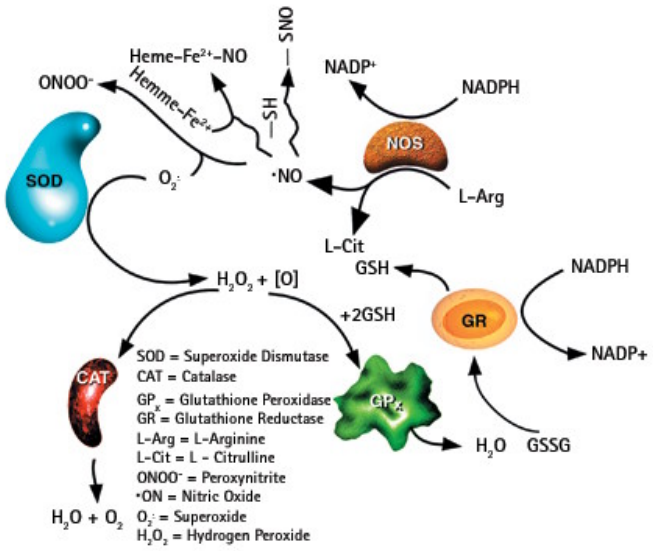


Fig 3 Oxidative stress pathway

Antioxidant agents

A predominant toxic effect of CM on renal tubules has been shown in both clinical trials and animal experiments [4-6]. Furthermore, administration of compounds with antioxidant properties such as N-acetylcysteine (NAC), ascorbic acid, and sodium bicarbonate has emerged as an effective strategy to prevent CIN [7,25-28]. Little is known about cellular mechanisms underlying CIN, and, as a consequence, about the mechanisms for the protective effect of compounds, such as NAC, ascorbic acid, and sodium bicarbonate.

An antioxidant is a molecule capable of slowing or preventing the oxidation of other molecules. Oxidation is a chemical reaction that transfers electrons from a substance to an oxidizing agent. Antioxidants terminate these chain reactions by removing free radical intermediates, and inhibit other oxidation reactions by being oxidized themselves. As a result, antioxidants are often reducing agents.

Although oxidation reactions are crucial for life, they can also be damaging; hence, plants and animals maintain complex systems of multiple types of antioxidants, such as glutathione, vitamin C, and vitamin E as well as enzymes such as catalase, superoxide dismutase and various peroxidases. Low levels of antioxidants, or inhibition of the antioxidant enzymes, causes oxidative stress and may damage or kill cells.

N-AcetylCysteine (NAC)

N-AcetylCysteine (NAC, N-Acetyl-L-Cysteine) (Fig 4) is the amino acid L-Cysteine plus an acetyl (-CO-CH_3) group attached to the amino (NH_2) group. The acetyl group functions to speed absorption and distribution upon orally cysteine . Amino acids which contain a sulfur group have antioxidant properties.

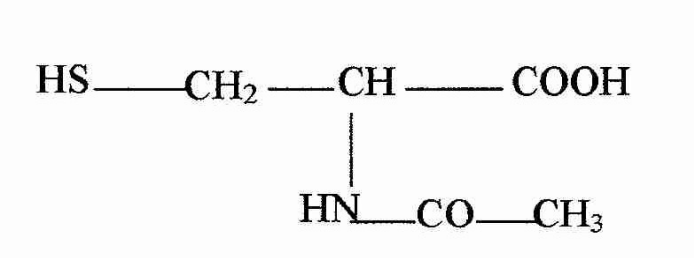


Fig. 4. N-acetylcysteine chemical structure

N-acetylcysteine (NAC) reduces the nephrotoxicity induced by constra media through antioxidant effects. It also enhances the effect of endogenous vasodilator nitric oxide. Some studies revealed successful protective effects of NAC as an adjunct to saline hydration in low-risk patients [29].

Ascorbic acid

Ascorbic acid is a sugar acid with antioxidant properties. Its appearance is white to light-yellow crystals or powder. It is water-soluble. The L-enantiomer of ascorbic acid is commonly known as vitamin C (fig 5). Ascorbate acts as an antioxidant by being available for energetically favourable oxidation. Reactive oxygen species oxidize ascorbate first to monodehydroascorbate and then dehydroascorbate. The reactive oxygen species are reduced to water, while the oxidized forms of ascorbate are relatively stable and unreactive, and do not cause cellular damage

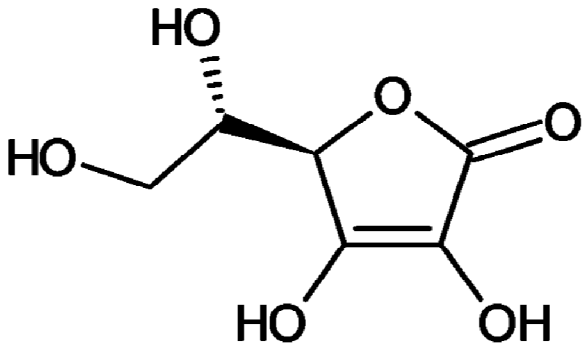


Fig. 5 Ascorbic Acid chemical structure

Sodium bicarbonate

Sodium bicarbonate or sodium hydrogen carbonate is the chemical compound with the formula $NaHCO_3$ (Fig 6). Sodium bicarbonate is a white solid that is crystalline but often appears as a fine powder. It is a component of the mineral natron and is found dissolved in many mineral springs. The natural mineral form is known as nahcolite. It is also produced artificially.

Sodium bicarbonate has antioxidant effects and scavenging reactive free radicals. In kidney environment this effect, it also decreases the acidification of urine and renal medulla, which may reduce the generation of free radicals and protects the kidney from oxidant injury [30].

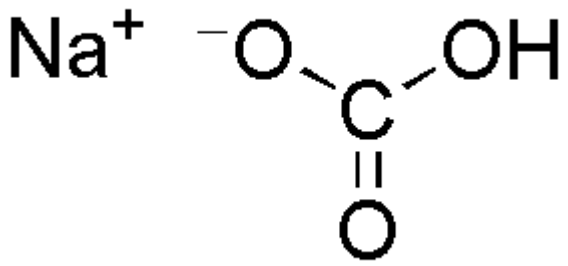


Fig 6. Sodium Bicarbonate chemical structure

Physicochemical Properties of Contrast Agents

The goal of producing contrast media that combine high attenuation with reduced side effects has resulted in a diverse family of compounds, each with differing physicochemical properties and physiologic effects. Agents such as diatrizoate and others were manufactured as polar (ionic) and highly concentrated solutions (high osmolarity) in order to ensure good visualization and water solubility. Although these high-osmolar contrast media (HOCM) allowed good opacification of small structures, they were found to be associated with significant pseudoallergic reactions and a significant risk of nephrotoxicity in patients at risk. Subsequent compounds (low-osmolar contrast media [LOCM]) had an osmolarity 2–3 times lower than HOCM; most compounds in this group, including iohexol and iopamidol, are nonionic, with ioxaglate as the only ionic agent. Although these agents were a definite improvement, the osmolarity was significantly greater than that of plasma and they were still associated with the development of CIN in a lower proportion of patients at risk. In patients at increased risk for CIN undergoing intra-arterial administration of contrast, ionic high-osmolality agents pose a greater risk for CIN than low-osmolality agents. Current evidence suggests that for intra-arterial administration in high-risk patients with chronic kidney disease, particularly those with diabetes mellitus nonionic, iso-osmolar contrast is associated with the lowest risk of CIN. The CIN Consensus Working Panel considered that there is

insufficient information to make a definitive statement about the relative contributions to renal toxicity. However, it seems clear that the osmolarity of the contrast medium, and hence the osmotic load delivered to the kidneys, appears to play a critical role in the pathogenesis of CIN, either directly or indirectly [18,23].

Pharmacokinetic properties of the Contrast Media.

Contrast agents are highly water-soluble and their active compound is distributed very rapidly througt the interstitial fluid. There is no evidence that CM molecules can penetrate plasma membrane, with the exception of renal tubule cells. The dimers increase the viscosity of fluids than the monomers. This may have relevance only in the proximal tubular lumen, where an increase in viscosity leads to a longer retention of CM and increased exposure of renal tubular cells to CM. The half-life of distribution is very fast [31].

The MC (high or low osmolarity) are iperosmolar compared to plasma and they generate a rapid passage of fluid from interstitial compartment to the vascular one and an increase in plasma volume. This phenomenon is transient, is proportional to the osmolarity of solution of CM and it lasts a few seconds. Water-soluble organic iodates are quickly eliminated by glomerular filtration. Their clearance in a person with normal renal function is 3 hours.

Classification of Contrast Media.

The iodinated contrast agents are different in their physical and chemical features, and they can be classified according to:

a) the osmolality of the solution (high osmolality, low osmolality or iso-osmolality),

b) dissociation in solution (ionic, non-ionic),

c) number of benzene rings (monomers, dimers).

The commonly contrast media used can be distinguished into four groups:

1) monomers, ionic, high osmolality. They are hyper-osmolar (about 1500 mOsm / kg H2O, ~ 7 times the plasma osmolality). They contain 3 iodine atoms for every 2 molecules produced in solution (which involves the common definition of MDC Class 1.5 or ratio 1.5:1). MDC are older and less expensive to use in clinical practice.

2) monomers, non-ionic low osmolality. Depending on the iodine concentration of non-ionic monomers, the osmolarity of these CM can range from 300 to 915 mOsm / kg H2O. They contain 3 iodine atoms for one molecule (which involves the establishment of MC common grade 3 or 3:1 ratio).

3) dimer, ionic, low osmolality. The molecule is composed of two tri-iodinated benzene rings (anion) and cations (sodium and meglumine).

The anion is a dimer, which contains 6 iodine atoms. When the iodine complex is dissociated, it's generates 2 particles: a cation without iodine, and an anion with 6 atoms of iodine. The effect is a compound with a ratio of 3 atoms for every iodine molecule in solution, with an osmolality similar to that of non-ionic solution monomers.

4) dimer, non-ionic iso-osmolality. These molecules are composed of two tri-iodinated benzene rings not dissociated in solution. The osmolarity of all non-ionic dimmers based solutions is `similar to plasma (290-300 mOsm/kg H2O). Each molecule of this family has 6 iodine atoms (which involves the common definition of CM (Class 6 or 6:1 ratio). These kind of CM molecules are larger than the previous. Thus their viscosity is higher than that of non-ionic monomers.

Based on this formula, we also can classify contrast agents:

$$\text{CLASS} = \frac{\text{Iodine atoms}}{\text{N° solution paticle}}$$

Table 1: Classification of Contrast Media

GROUP	IODINE ATOMS	N° PARTICLE	CLASS
Ionic monomer	3	2	1.5
Non-ionic monomer	3	1	3
Ionic dimer	6	2	3
Non-ionic dimer	6	1	6

Iodixanol

It is a contrast agent, sold under the trade name Visipaque (class 6). Visipaque is commonly used as a contrast agent during coronary angiography, particularly in individuals with renal dysfunction, as it is believed to be less toxic to the kidneys than most other intravascular contrast agents. It is an iso-osmolar contrast agent, with an osmolality of 290 mOsm/kg H2O, the same as blood (fig 7).

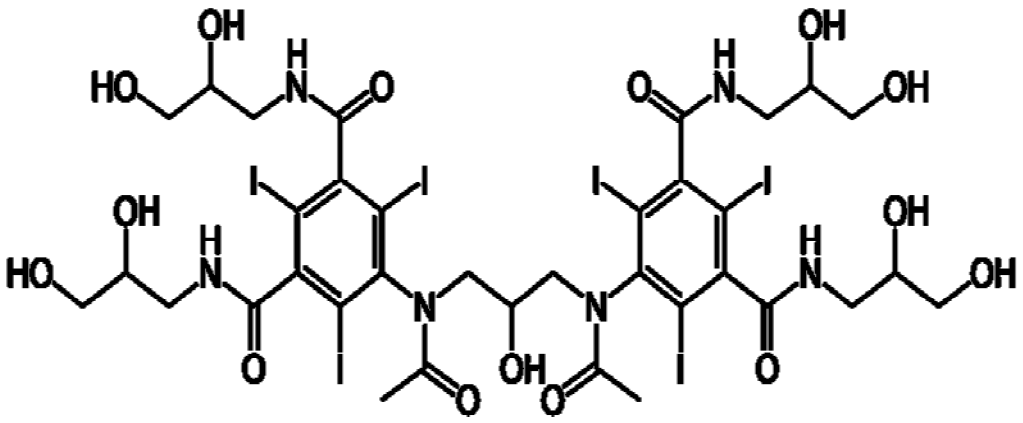


Fig 7 Iodixanol chemical structure

Iobitridol

Is a nonionic low-osmolar class 3 contrast medium approved and marketed in Germany as Xenetix (Guerbet, Sulzbach, Germany) since 1996. Xenetix is commonly used as a contrast agent during coronary angiography. It is an iso-osmolar contrast agent, with an osmolality of 915 mOsm/kg H2O, lower than blood osmolarity (fig 8).

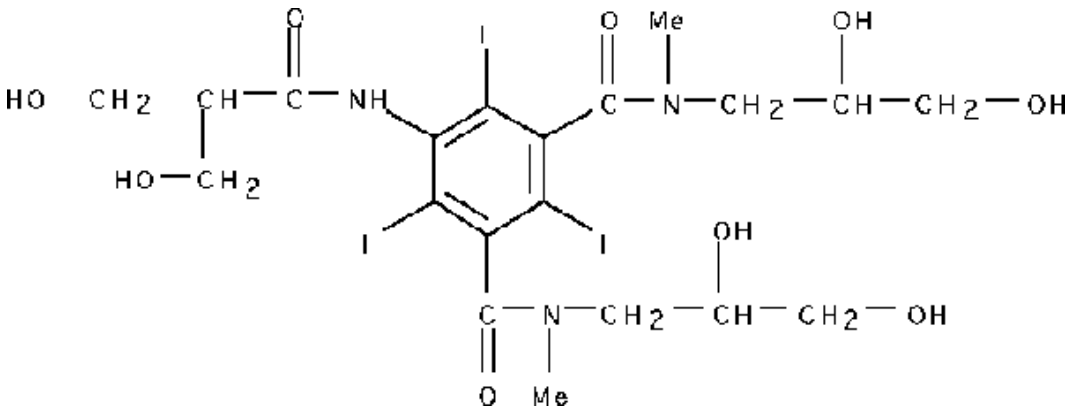


Fig 8 Iobitridol chemical structure

Apoptosis

The word *Apoptosis* has greek origin, means "falling off or dropping off", in analogy to leaves falling off trees or petals dropping off flowers. This analogy emphasizes the death is an integral and necessary part of the life cycle of organisms. The apoptotic mode of cell death is an active and defined process which plays an important role in the development of multicellular organisms and in the regulation and maintenance of the cell populations in tissues upon physiological and pathological conditions [32].

During development many cells are produced in excess and eventually undergo programmed cell death and thereby contribute to sculpturing many organs and tissues [33].

Taken together, apoptotic processes have widespread biological significance, being involved in e.g. development, differentiation, proliferation/homoeostasis, regulation and function of the immune system and in the removal of defect and therefore harmful cells. Thus, dysfunction or dysregulation of the apoptotic program is implicated in a variety of pathological conditions [34].

Apoptotic cells can be recognized by stereotypical morphological changes: the cell shrinks, shows deformation and looses contact to its neighbouring cells. Its chromatin condenses and marginates at the nuclear membrane, the plasma membrane is blebbing or budding, and

finally the cell is fragmented into compact membrane-enclosed structures, called 'apoptotic bodies' which contain cytosol, the condensed chromatin, and organelles. Those morphological changes are a consequence of characteristic molecular and biochemical events occurring in an apoptotic cell, most notably the activation of proteolytic enzymes which eventually mediate the cleavage of DNA into oligonucleosomal fragments as well as the cleavage of a multitude of specific protein substrates which usually determine the integrity and shape of the cytoplasm or organelles [35].

Principally two mechanisms have been identified in mammalian cells for the induction of apoptosis: agents that lead to the perturbation of mitochondria, resulting in the release of cytochrome C and the activation of apoptosis; or agents that directly activate a family of death receptors leading to the activation of a parallel apoptotic cascade [36] (Fig 9).

The caspases, cysteine proteases homologous to *C. elegans* ced-3 are of central importance in the apoptotic signalling network which are activated in most cases of apoptotic cell death [37].

The term caspases is derived from cysteine-dependent aspartate-specific proteases: their catalytical activity depends on a critical cysteine-residue within a highly conserved active-site pentapeptide QACRG and the caspases specifically cleave their substrates after Asp residues.

The observation that cell lines derived from those knockout experiments are resistant to distinct apoptosis stimuli underlines the

importance of caspases as proapoptotic mediators. Indeed, caspase-3, caspase-9, caspase-8, and additionally caspases-2, -6, -7, and –10 have been recognized to play an important role in the apoptotic signalling machinery [38].

The proapoptotic caspases can be divided into initiator caspases including procaspases-2, -8, -9 and –10, and into the group of executioner caspases including procaspases-3, -6, and –7. Whereas the executioner caspases possess only short prodomains, the initiator caspases possess long prodomains, containing death effector domains (DED) in the case of procaspases-8 and –10 or caspase recruitment domains (CARD) as in the case of procaspase-9 and procaspase-2.
Via their prodomains, the initiator caspases are recruited to and activated at death inducing signaling complexes either in response to the ligation of cell surface death receptors (extrinsic apoptosis pathways) or in response to signals originating from inside the cell (intrinsic apoptosis pathways). In extrinsic apoptosis pathways, e.g. procaspase-8 is recruited by its DEDs to the death inducing signalling complex (DISC), a membrane receptor complex formed following to the ligation of a member of the tumor necrosis factor receptor (TNFR) family [39].

When bound to the DISC, several procaspase-8 molecules are in close proximity to each other and therefore are assumed to activate each other by autoproteolysis [40].

Intrinsic apoptosis pathways involve procaspase-9 which is activated downstream of mitochondrial proapoptotic events at the so called apoptosome, a cytosolic death signalling protein complex that is formed upon release of cytochrome c from the mitochondria [41].

In this case it is the dimerization of procaspase-9 molecules at the Apaf-1 scaffold that is responsible for caspase-9 activation [40].

Once the initiator caspases have been activated, they can proteolytically activate the effector procaspases-3, -6, and -7 which subsequently cleave a specific set of protein substrates, including procaspases themselves, resulting in the mediation and amplification of the death signal and eventually in the execution of cell death with all the morphological and biochemical features usually observed [38].

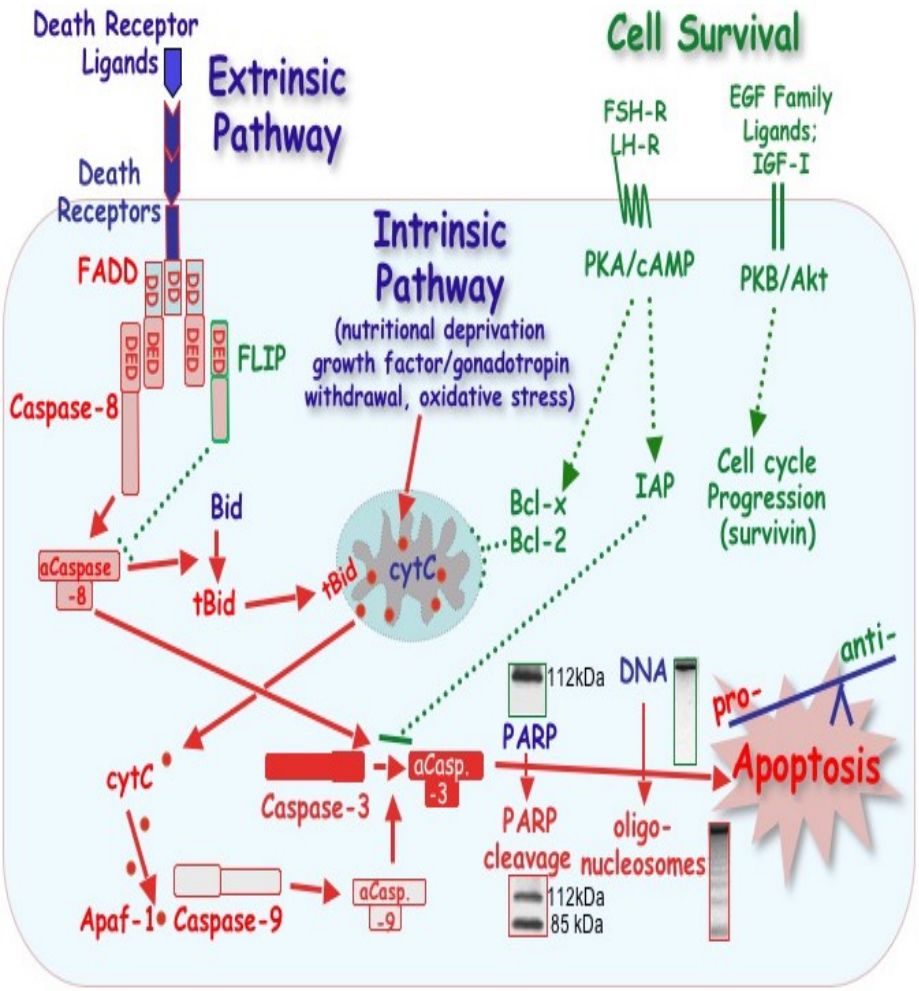


Fig 9. Simplified model of pro- and anti-apoptotic pathways.

Aim of study

The aim of the study is to investigate the molecular mechanisms underlying the apoptotic effects of both iso-osmolar (IOCM) and low-osmolar (LOCM) CM on patients kidneys, by testing the response of non-differentiated human embryonic kidney cells (HEK 293), and of differentiated cells, i.e. porcine proximal renal tubular cells (LLC-PK1) and canine Madin-Darby distal tubular renal cells (MCDK) after CM treatment. Futhermore, we aimed to determine the role of commonly used antioxidant compounds in clinical investigation on preventing CM-induced apoptosis.

Experimental Procedures

Culture conditions and reagents

Three different cell lines were utilized: (i) human embryonic kidney (HEK 293), which are undifferentiated human renal cells; (ii) porcine proximal renal tubular (LLC-PK1) and canine Madin–Darby renal epithelial (MDCK) cells which have the characteristics of proximal and distal tubule cells, respectively. Cells were grown in a 5% CO_2 atmosphere in Dulbecco's Modified Eagle Medium (DMEM) containing 10% heat-inactivated FBS, 2 mM L-glutamine, and 100 U/mL penicillin–streptomycin. Cells were routinely splitted when they reached 80–85% confluent. Media, sera, and antibiotics for cell culture were from Life Technologies, Inc. (Grand Island, NY, USA). Protein electrophoresis reagents were from Bio-Rad (Richmond, VA, USA) and western blotting and ECL reagents (GE Health care, Europe SA). All other chemicals were from Sigma (St Louis, MO, USA).

Contrast agents

Two different CM were tested: (i) iodixanol (Visipaque®, GE Healthcare Europe; 320 mg iodine/mL) non-ionic, IOCM (290 mOsm/kg of water) and (ii) iobitridol (Xenetix®, Guerbet, France; 250 mg iodine/mL) non-ionic, LOCM (915 mOsm/kg of water).

Experimental design

Experiments were driven in the following phases: (i) assessment of cytotoxicity of both LOCM (iobitridol) and IOCM (iodixanol). In order to assess the impact of contrast dose, two different doses of CM were tested, 100 and 200 mg iodine/mL. The cytotoxicity of CM was tested at 15, 30, 45, 60, 90, 120, 150, 180. The osmolality of DMEM alone was 355 mOsm/L, when compared with 395 mOsm/L for DMEM plus IOCM and 830 mOsm/L for DMEM plus LOCM. In order to clarify the potential major determinants of the cytotoxic effect, we further assessed the effect of iodine alone (by incubation with 100 and 200 mg/mL sodium iodine) [15] and hyperosmolality (by incubation in DMEM/8% mannitol, having an osmolality of 830 mOsm/L); (ii) assessment of the effectiveness of various antioxidant compounds (that is, NAC, ascorbic acid, and sodium bicarbonate) in preventing contrast cytotoxicity. Different doses of all tested compounds were utilized, in order to elicit any dose-dependent effect. The doses tested were selected according to the available data in the clinical setting. NAC was tested at 1, 10, and 100 mM [16,17]. Ascorbic acid was tested at 2, 4, and 8 mM [42]. Sodium bicarbonate was tested at 75, 150, and 300 mM [27]. Each concentration was done in triplicate.

Protein isolation and western blotting

Cellular pellets from a singular cell line at time were washed twice with cold PBS and resuspended in JS buffer (HEPES 50 mM, NaCl 150 nM, 1% glycerol, 1% Triton X-100, 1.5 mM $MgCl_2$, 5 mM EGTA) containing Proteinase Inhibitor Cocktail (Roche). Solubilized proteins were incubated for 1 h on ice. After centrifugation at 13 200 rpm for 10 min at 4°C, lysates were collected as supernatants. Eighty micrograms of sample extract were resolved on a 12% SDS-polyacrylamide gel using a mini-gel apparatus (Bio-Rad Laboratories, Richmond, CA, USA) and transferred to Hybond-C extra nitrocellulose (GE Healthcare Europe). Membrane was blocked for 1 h with 5% non-fat dry milk in TBS containing 0.05% Tween-20 and incubated over night at 4°C with specific antibodies. The following antibodies were used for immunoblotting: anti-pro-caspase-3 (recognizing only the inactive pro-caspase-3) (cell signalling), anti-beta Actin (Sigma), anti-PARP (Sigma), anti-Bim (Santa Cruz), anti Bad (Santa cruz), and anti-Caspase-9, -10, and -8 from Stressgen. Washed filters were then incubated for 60 min with HRP-conjugated anti-rabbit or anti-mouse secondary antibodies (GE Healthcare, Europe) and visualized using chemioluminescence detection (GE Healthcare Europe). The activation of caspase was followed by the disappearance of the band corresponding to the inactive pro-caspase enzyme, utilizing a specific antibody that recognizes this form.

Cell-death quantification

Cell death was evaluated with the CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay (Promega, Madison, WI, USA), according to the manufacturer's protocol. The assay is based on reduction of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) to a coloured product that is measured spectrophotometrically. Cells were plated in 96-well plates in triplicate, stimulated, and incubated at 37°C in a 5% CO_2 incubator. Iobitridol, iodixanol, NAC, sodium bicarbonate, and ascorbic acid were used *in vitro* at the doses and time indicated. Metabolically active cells were detected by adding 20 µL of MTS to each well. After 30 min of incubation, the plates were analysed on a Multilabel Counter (Bio-Rad, Richmond, VA, USA). DNA laddering was also used to confirm the apoptotic death induced by CM. Briefly, after CM exposure, the cells were harvested with 500 µL of DNA lysis buffer [5 mM Tris–HCl (pH 7.5), 20 mM EDTA (pH 8.0), 0.5% NP40], and were incubated on ice for 20 min. After centrifugation at 13 200 rpm for 30 min, the DNA was then extracted with phenol chloroform isoamyl alcohol and finally precipitated with the addition of 1.25 mL of cold ethanol 100% and 50 µL sodium acetate (pH 5.2) on dry ice for 20 min. The precipitates were centrifuged (30 min, 13 200 rpm, 4°C), dried at room temperature, solubilized in 10 µl of TE, and then incubated with RNase A for 30 min at 37°C. The DNA samples were finally separated on 1.5% agarose gel containing ethidium bromide (Sigma, St Louis). The gel was photographed under UV light. Apoptosis was also analysed via propidium iodide

incorporation in permeabilized cells by flow cytometry. The cells (2 x 10^5) were washed in PBS and resuspended in 200 µL of a solution containing 0.1% sodium citrate, 0.1% Triton X-100, and 50 µg/mL propidium iodide (Sigma). Following incubation at 4°C for 30 min in the dark, nuclei were analysed with a Becton Dickinson FACScan flow cytometer. Cellular debris was excluded from analyses by raising the forward scatter threshold, and the DNA content of the nuclei was registered on a logarithmic scale. The percentage of elements in the hypodiploid region was calculated.

Statistical analysis

Continuous variables are expressed as mean values ± SD. We performed a multiple comparison test using the information derived by performing one-way analysis of variance (ANOVA) test on groups of independent variables having cell viability as our dependent variable. In an ANOVA, we compared the means of several groups to test the hypothesis that they are all the same, against the general alternative that they are not all the same. However, since the alternative hypothesis may be too general and more information is needed about which pairs of means are significantly different, and which are not, we used the multiple comparison procedure, which allows us to comparing all group mean pairs at the same time. Throughout the analysis, we have specified a significance level α= 0.001 and we performed priori comparisons on the outputs derived from ANOVA test. Also, main focus was given on the ANOVA outputs where the *F*

test resulted significantly. We performed the priori comparisons using the Bonferroni *t* method for both orthogonal and non-orthogonal comparisons to reduce multiplicity between group comparisons. The Bonferroni *t* method increases the critical *F* value needed for the comparison to be declared significant. Data were analysed with SPSS 13.0 (Chicago, IL, USA) for Windows.

RESULTS

Effects of contrast medium on cell viability

As shown in *Figure 10*, both LOCM and IOCM produced a concentration-dependent decrease in cell viability as assessed by MTS assay. This effect was identical in all the three renal cell lines utilized (*Figure 10*). The toxic effect of CM was further evaluated by DNA laddering (*Figure 11*) and propidium staining and FACS analysis (*Figure 12*). Both methods confirmed that exposure of cells to LOCM and IOCM induces apoptosis of renal cells.

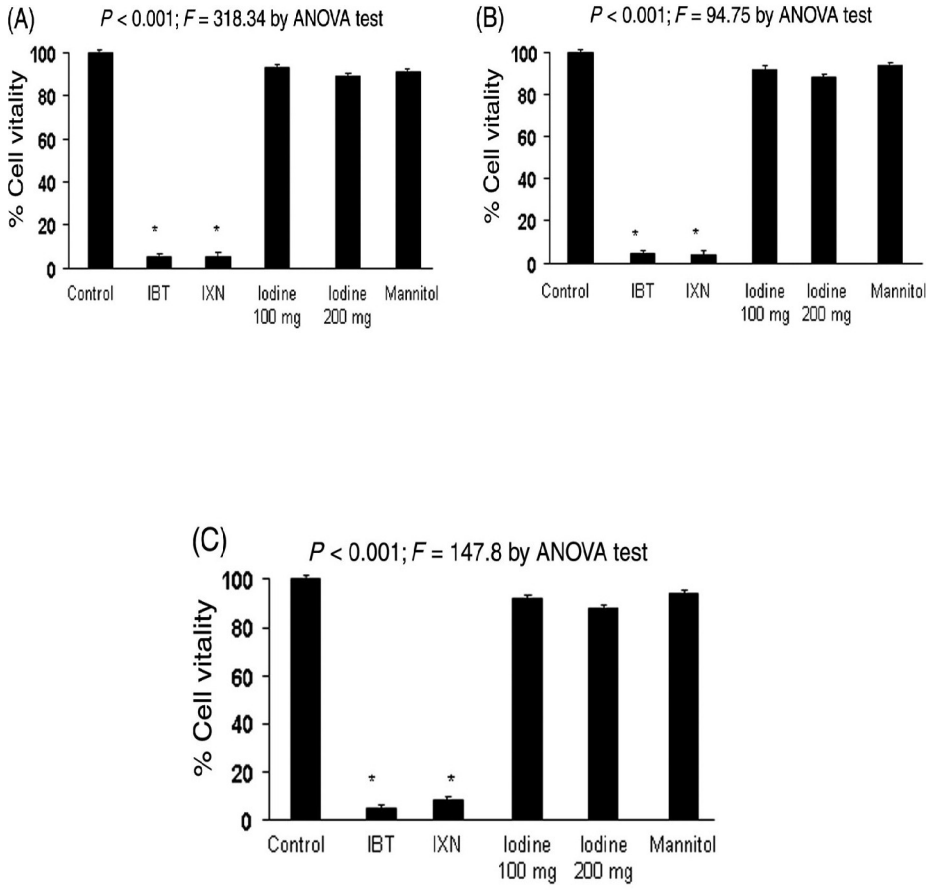


Figure 10 Effects of contrast media, iodine, and mannitol on renal cells. HEK 293 (*A*), LCC-PK1 (*B*), and MCDK (*C*) cells were incubated in the presence of 200 mg iodine/mL of iobitridol (IBT) or iodixanol (IXN), of iodine alone (sodium iodine, 100 and 200 mg) and 8% mannitol (hyperosmolar solution) for 3 h. Cell viability was then assessed with CellTiter Proliferation Assay. **P* < 0.001 vs. all the other groups;

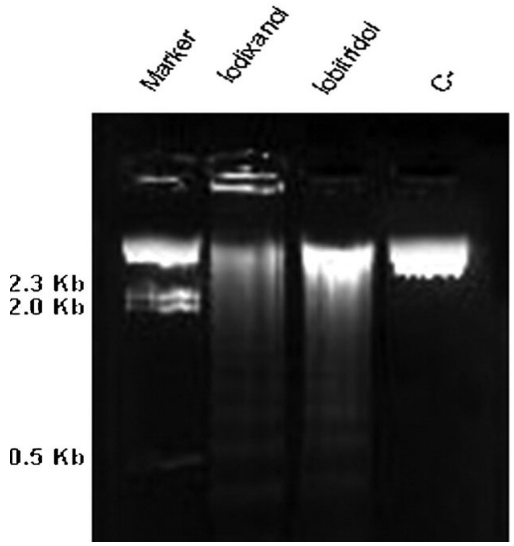


Fig 11 HEK 293, LCC-PK1, and MDCK cells were incubated in the presence of 200 mg iodine/mL of iobitridol or iodixanol for 3 h. DNA was extracted and loaded on 1.5% agarose gel.

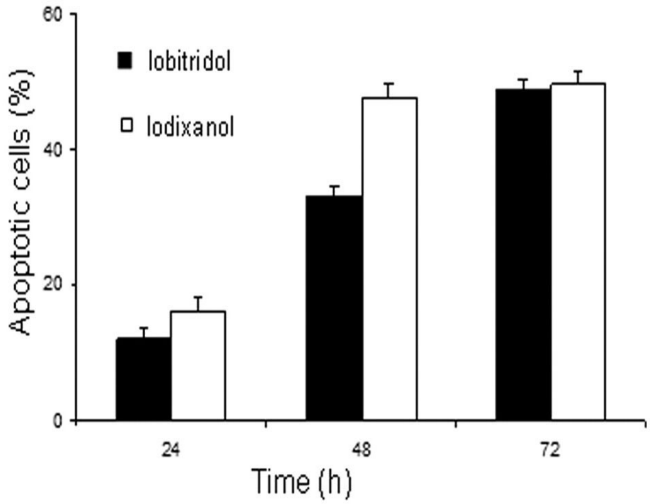
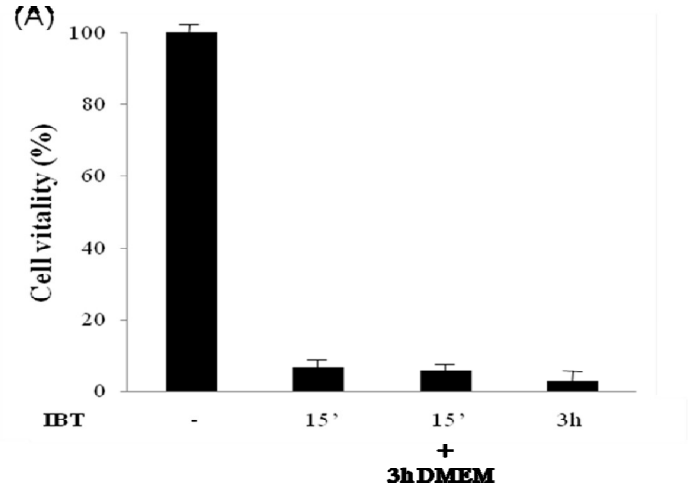


Fig 12 HEK 293, LCC-PK1, and MDCK cells were incubated in the presence of 100 and 200 mg iodine/mL of iobitridol or iodixanol for 24, 48, or 72 h and then DNA fragmentation was measured by flow cytometry. Data represent the mean±SD of two separate experiments performed in triplicate.

The cytotoxic effect, although maximum at 3 h, was mostly (≈85%) observed already at 15 min of incubation. In order to better clarify the time-dependency effect, we performed a further control experiment in which cells were exposed for a short period (only 15 min), then washed free of CM, and studied for viability immediately or 3 h later, and compared these effects to those observed upon 3 h of incubation. Interestingly, we found that the cytotoxic effect induced by 15 min of high dose (200 mg iodine/mL) of CM exposure was similar whether it was observed immediately or 3 h later *(Figure 13)*

There was not any interaction between the cytotoxic effect and the type of contrast used (P = 0.22; F = 1.87 by ANOVA model with α= 0.001). Furthermore, neither sodium iodine alone nor hyperosmolar solution decreased cell viability or induced cell apoptosis (*Figure 14*).



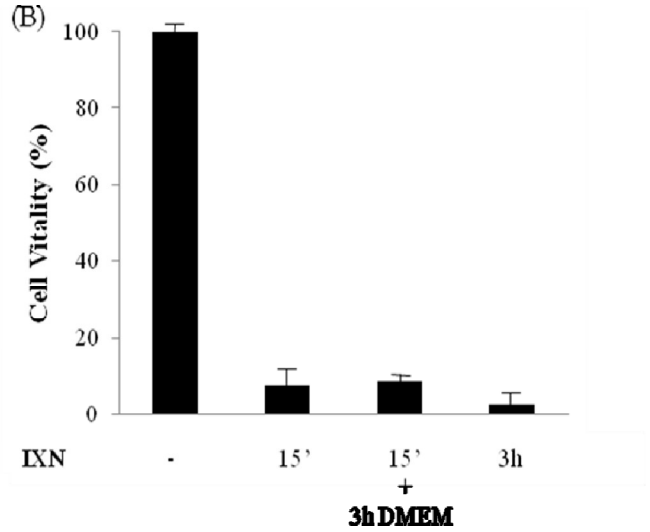


Figure 13 Role of different time on effect of contrast media. MCDK cells were incubated in the presence of 200 mg iodine/mL of iobitridol (A) or iodixanol (*B*), for 15 min, 3 h or 15 min then washed and incubated with DMEM, respectively. Data represent the mean±SD of two separate experiments performed in triplicate.

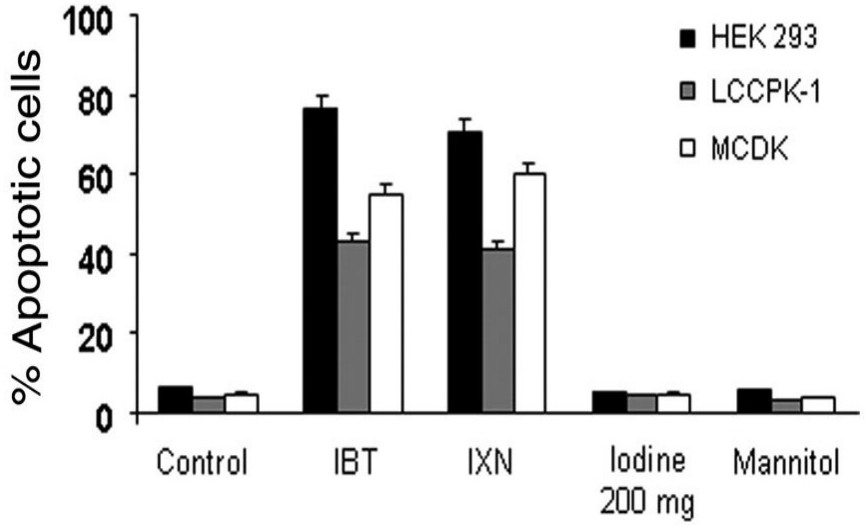


Fig. 14 HEK 293, LCC-PK1, and MDCK cells were incubated in the presence of 200 mg iodine/mL of iobitridol or iodixanol for 72 h and then DNA fragmentation was measured by flow cytometry. Data represent the mean±SD of two separate experiments performed in triplicateAll cell lines were exposed to the same concentrations of CM. On the contrary, *in vivo*, cell apoptosis was mostly found in the more distal tubular cells (MDCK) which may be exposed to higher concentrations of CM.

Role of caspases in contrast-induced cytotoxicity

To test whether CM stimulate caspase activity, HEK 293 cells were incubated in the presence of either LOCM or IOCM at different time points and then the activation of caspases-8, -10, -3, -9 was assessed by western blot (*Figure 14A*). Both LOCM and IOCM caused a marked increase in caspase-3 and -9 activities at 7 h of exposure, as assessed by the reduction of the pro-caspase form (*Figure 15A*). No effect on caspases-8 and -10 was observed, thus indicating that the CM activated apoptosis mainly through the intrinsic, or 'mitochondrial', pathway (*Figure 14A*). This pathway of apoptosis is regulated by Bcl2 family members. Hence, we studied the expression of Bad and Bim, two pro-apoptotic members of the Bcl2 family, after incubation with the CM. Western blotting revealed that exposure to CM induce an increase in both Bad and Bim expression (*Figure 15B*). Similar results were obtained in the other cell types (data not shown).

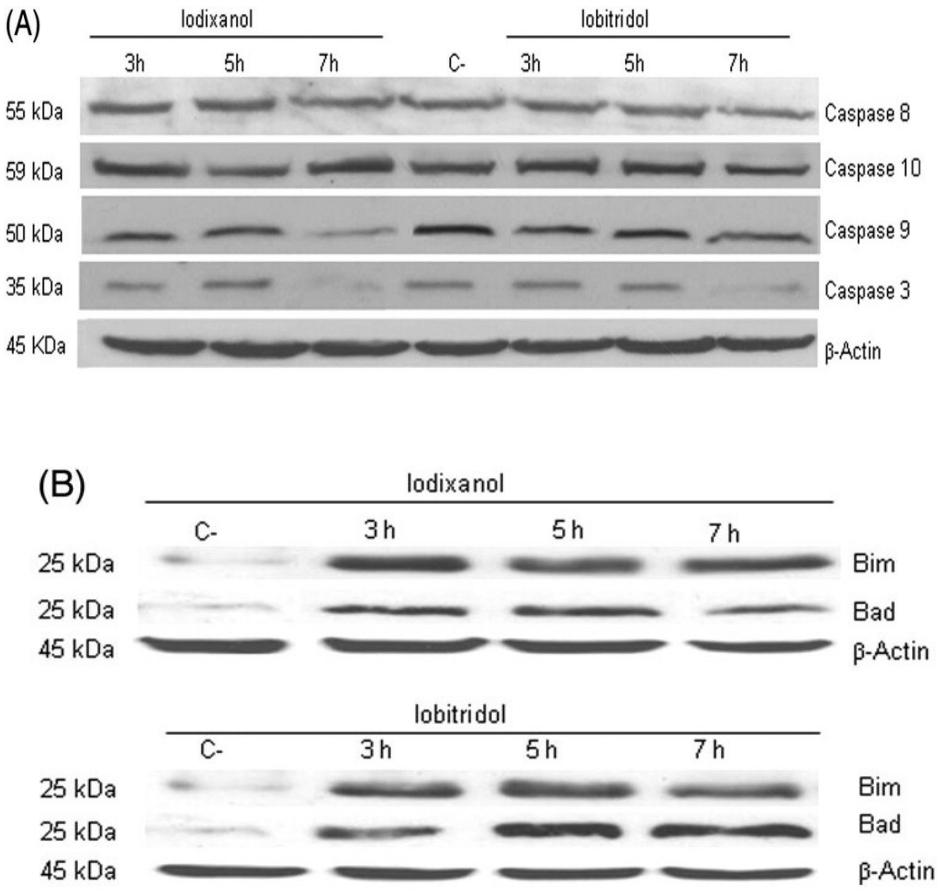


Figure 15 Effect of contrast media on caspase activation and on Bcl2 family proteins expression. HEK 293 cells were incubated in the presence of 200 mg iodine/mL of iobitridol or iodixanol for the indicated time. Eight micrograms of protein were loaded onto 12% SDS–PAGE gel. (*A*) The membranes were incubated with anti-caspase-3, -8, -9, -10 antibodies and visualized by chemioluminescence detection. Contrast media induced an activation of caspases-3 and -9 and not of caspases-8 and -10 as assessed by the reduction of the pro-caspase levels. (*B*) Membranes were incubated with anti-Bim or anti-Bad antibodies and visualized by chemioluminescence detection. Loading control was controlled with anti-β-actin.

Effects of NAC on contrast-induced cytotoxicity

HEK 293, LLC-PK1, and MDCK cells were pre-incubated with different concentrations of NAC and cell viability was assessed with the cell proliferation assay. We observed a dose-dependent protective effect of NAC on renal cells after 3 h incubation with the high dose (200 mg iodine/mL) of both LOCM and IOCM ($P < 0.001$; $F = 396.22$ by ANOVA test; (*Figure 16*). As compared to baseline, after 3 h of incubation, cell viability was <10% in the CM-treated cells, <25% with the lowest (1 mM) dose of NAC, <30% with the middle (10 mM), and approximately 80% with the highest (100 mM) dose of NAC. There was not any interaction between the protective effect of NAC (for dose 1 and 10 mM) and the type of CM ($P = 0.75$; $F = 0.12$ and $P = 0.32$; $F = 1.31$, respectively, both by ANOVA test with $\alpha = 0.001$). However, results for NAC 100 mM with LOCM appears to be slightly better for cell viability when compared with NAC 100 mM with IOCM ($P = 0.006$; $F = 28.22$). In order to clarify the mechanism by which NAC prevented contrast-induced apoptosis, we analysed the effect of NAC pre-treatment on Poly(ADP-ribose) (PARP), a final substrate of caspase-3. We found that the CM induced the activation of PARP as assessed by the marked reduction of the 116 kDa PARP pro-form. On the contrary, NAC completely prevented this activation, suggesting that NAC acts through the inhibition of the intrinsic pathway of apoptosis (*Figure 17*).

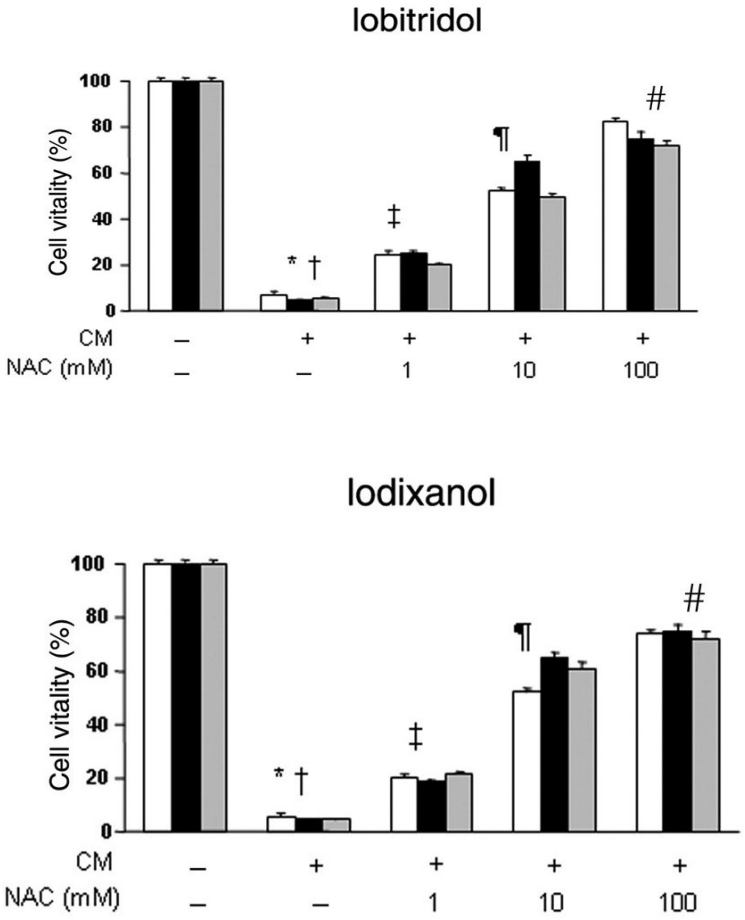


Figure 16 Pre-treatment with NAC and contrast-induced cell death. HEK 293 (open columns), LCC-PK1 (black columns), and MDCK (grey columns) cells were pre-treated for 2 h with different concentration of N-acetylcysteine (NAC) as indicated (1, 10, and 100 mM) and then incubated for 3 h with 200 mg iodine/mL of iobitridol or iodixanol. Cell viability was then assessed with CellTiter Proliferation Assay. NAC protects the cells from contrast-induced cell death in a dose-dependent fashion (HEK 293: $P < 0.001$; $F = 143.51$ with iobitridol; $P < 0.001$; $F = 122.43$ with iodixanol. LCC-PK1 cells: $P < 0.04$; $F = 12.9$ with iobitridol; $P < 0.03$; $F = 14.79$ with iodixanol; MDCK cells: $P < 0.006$; $F = 41.19$ with iobitridol; $P < 0.01$; $F = 25.24$ with iodixanol). $^{*}P = <0.001$ vs. baseline (CM–/NAC–) and [CM+/NAC 100 mM]; $^{\dagger}P < 0.05$ vs. (CM+/NAC 1 mM) and (CM+/NAC 10 mM); $^{\ddagger}P = 0.003$ vs. (CM+/NAC 100 mM); $^{\P}P < 0.03$ vs. (CM+/NAC 100 mM); $^{\#}P < 0.05$ vs. baseline (CM–/NAC–).

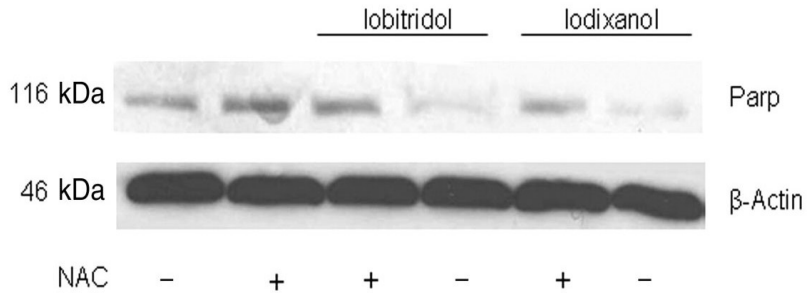


Fig 17. HEK 293 cells were pre-incubated with NAC for 2 h and then incubated in the presence of 200 mg iodine/mL of iobitridol or iodixanol for 3 h. Eighty microgram of protein was loaded onto 10% SDS–PAGE. The membranes were incubated with anti-PARP antibody and visualized by chemioluminescence detection. PARP activation was detected by the reduction of the 116 kDa non-cleaved form of PARP. Loading was controlled with anti-b-actin.

Effects of ascorbic acid on contrast-induced cytotoxicity

We observed a dose-dependent protective effect of ascorbic acid on renal cells exposed after 3 h of incubation with the high dose (200 mg iodine/mL) of both LOCM (HEK 293: $P = 2.99 \times 10^{-5}$; $F = 1552.67$; LLC-PK1: $P = 0.04$; $F = 10.85$; MDCK: $P = 0.04$; $F = 18.57$) and IOCM (HEK 293: $P = 6.43 \times 10^{-5}$; $F = 933.55$; LLC-PK1: $P = 0.02$; $F = 16.29$; MDCK: $P = 0.01$; $F = 19.98$) (*Figure 18*). When compared to baseline, at 3 h of incubation cell viability was <6% in the control group, <15% with the lowest (2 mM) dose of ascorbic acid, and <60% with in both 4 mM and 8 mM doses of ascorbic acid, respectively. There was a significant interaction between the protective effect of ascorbic acid and cell viability for both types of CM (Iobitridol: $P = 0.0017$, $F = 10.09$, and Iodixanol: $P = 0.0002$, $F = 16.46$, both by the ANOVA model).

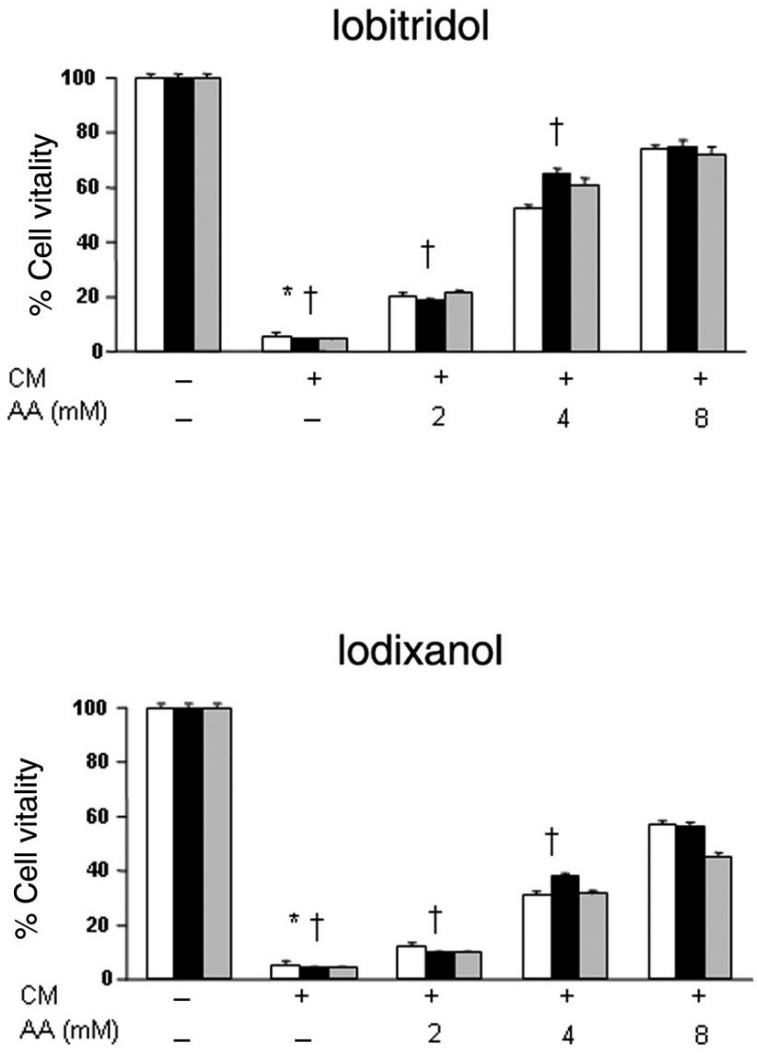


Figure 18. Effects of ascorbic acid on contrast-induced cell death. HEK 293 (open columns), LCC-PK1 (black columns), and MDCK (grey columns) were pre-treated for 2 h with different concentrations of ascorbic acid (AA) (2, 4, and 8 mM) or sodium bicarbonate (75, 150, and 300 mM) as indicated and then incubated for 3 h with 200 mg iodine/mL of iobitridol or iodixanol. Cell viability was then assessed with CellTiter Proliferation Assay. *$P < 0.03$ vs. (CM+/AA 2 mM+), †$P < 0.001$ vs. all the other groups.

Effects of sodium bicarbonate on contrast-induced cytotoxicity

We did not find any protective effect of sodium bicarbonate on HEK 293 (LOCM: $P = 0.53$; $F = 0.78$; IOCM: $P = 0.02$; $F = 23.02$); LCC-PK1 (LOCM: $P = 0.09$; $F = 6$; IOCM: $P = 0.94$; $F = 0.06$); and MDCK (LOCM: $P = 0.88$; $F = 0.13$; IOCM: $P = 0.71$; $F = 0.38$) after 3 h of incubation with the high dose of either LOCM or IOCM. Cell viability was quite similar even in the presence of high (300 mM) dose of sodium bicarbonate (*Figure 19*). This lack of any protective effect was similar with LOCM and IOCM. There was no difference in pH in the medium from the various groups (*Table 2*) and there was no effect on cell viability (Iobitridol: $P = 0.72$, $F = 0.33$; Iodixanol: $P = 0.49$, $F = 0.73$ by the ANOVA model).

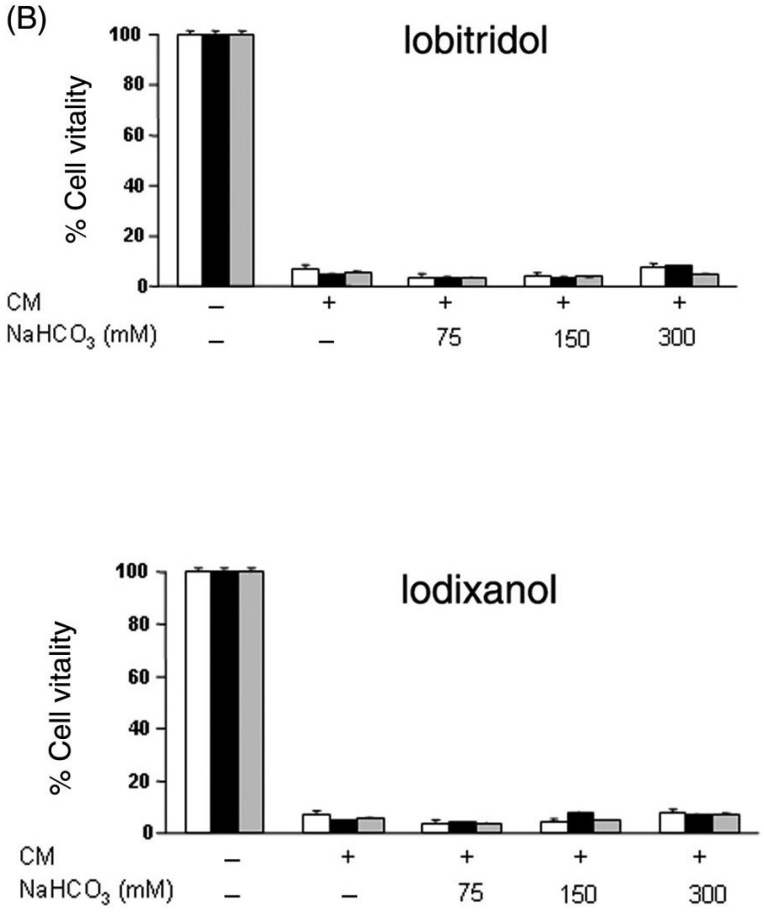


Figure 19 Effects of ascorbic acid and sodium bicarbonate on contrast-induced cell death. HEK 293 (open columns), LCC-PK1 (black columns), and MDCK (grey columns) were pre-treated for 2 h with different concentrations of ascorbic acid (AA) (2, 4, and 8 mM) or sodium bicarbonate (75, 150, and 300 mM) as indicated and then incubated for 3 h with 200 mg iodine/mL of iobitridol or iodixanol. Cell viability was then assessed with CellTiter Proliferation Assay. *$P < 0.03$ vs. (CM+/AA 2 mM+), †$P < 0.001$ vs. all the other groups.

Table 2 pH in the various treatment groups

Group	Iobitridol*	Iodixanol**
Contrast media alone	7.34 (7.18–7.50)	7.25 (7.06–7.50)
Contrast media plus NAC	7.29 (6.97–7.60)	7.03 (6.96–7.10)
Contrast media plus AA	6.90 (6.80–7.01)	7.04 (7.06–7.10)
Contrast media plus $NaHCO_3$	7.30 (7.21–7.40)	7.24 (7.08–7.50)

Values are expressed as median and interquartile range.

NAC, N-acetylcysteine; AA, ascorbic acid; $NaHCO_3$, sodium bicarbonate.

*P = 0.57 through the groups by ANOVA test, after transforming pH values into proton H^+ concentrations.

**P = 0.65 through the groups by ANOVA test after transforming pH values into proton H^+ concentrations.

Effects of co-incubation of NAC with ascorbic acid or with sodium bicarbonate

The protective effect of NAC (100 mM) was greater than that of ascorbic acid (8 mM) LOCM: $P = 1.25 \times 10^{-8}$, $F = 52.21$; and IOCM $P = 9.90 \times 10^{-9}$, $F = 54.03$ by the ANOVA model; (*Figure 20*). We performed a further experiment to investigate the effect on cell death of 2 h of NAC pre-treatment (100 mM), in the presence of either ascorbic acid (8 mM), or sodium bicarbonate (150 mM) on cell death after 3 h of incubation with the high dose (200 mg iodine/mL) of either LOCM or IOCM. As shown in *Figure 20*, the combination of NAC with another antioxidant agent was less effective than NAC alone ($P = 0.95$; $F = 0.54$ by the ANOVA test).

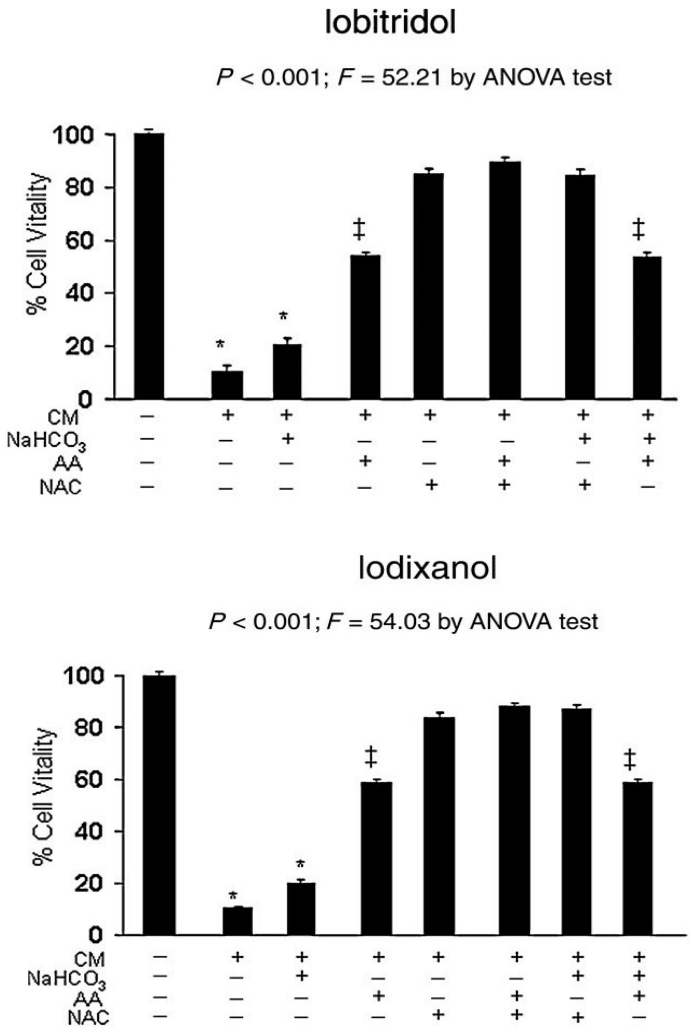


Figure 20 Effect of NAC, ascorbic acid, and sodium bicarbonate alone and in combinations on contrast-induced cell death. HEK 293 cells were pre-treated for 2 h in the presence of N-acetylcysteine (NAC, 100 mM), ascorbic acid (AA, 8 mM), and sodium bicarbonate ($NaHCO_3$, 150 mM) alone or in combination and then incubated for 3 h with 200 mg iodine/mL of iobitridol or iodixanol. Cell viability was then assessed with CellTiter Proliferation Assay. *P < 0.001 vs. (CM+/AA+), (CM+/NAC+), (CM+/NAC+/AA+), (CM+/NAC+/$NaHCO_3$+), and (CM+/AA+/$NaHCO_3$+); ‡P < 0.001 vs. (CM+/NAC+), (CM+/NAC+/$NaHCO_3$+), and (CM+/NAC+/AA).

Discussion

The main conclusions of the present study are (i) CM induce dose- and time-dependent renal cell apoptosis through the activation of the intrisinc pathway, (ii) this cytotoxic effect does not seem to be caused by iodine or osmolality ≤830 mOsm/L, and (iii) pre-treatment with NAC and ascorbic acid but not with sodium bicarbonate prevents apoptosis in a dose-dependent fashion.

Contrast media and renal cell apoptosis

Our study confirms that the CM induce renal cell apoptosis.[4–6,16–18] In order to strengthen this finding, we used three different renal cell lines, namely, human epithelial cells (HEK 293) and two cell lines with the characteristics of proximal and distal tubule cells [porcine kidney proximal tubular epithelial cells (LLC-PK1) and Madin–Darby canine kidney cells (MDCK)]. The activation of caspase-9 and -3, but not of caspases-8 and -10 observed after exposure to CM supports the concept that CM induce apoptosis through the intrinsic, or 'mitochondrial', pathway. This finding was also supported by the activation of PARP, a final substrate of caspase-3. In a rat model of CIN, cellular injury of the renal medulla consisted of extensive DNA fragmentation, which has been attributed to medullary hypoxia [43-45]. Yano *et al*. [45] have shown that CM induced apoptosis in the porcine tubular cell line, LLC-PK-1, and that the injuries might be due

to de-regulation in Bax/Bcl-2 expression, followed by increases in caspases-9 and -3 activities. In agreement with these previous observations, we found that CM induce an increase of at least two Bcl-2 pro-apoptotic family members, i.e. Bim and Bad [46-48].

Role of contrast dose and osmolality

CM induce renal cell apoptosis in a dose- and time-dependent manner.[18] Guidelines recommend to limit the volume of CM usage in order to prevent contrast-associated nephrotoxicity [49,50]. It has been suggested that using the iodine dose/glomerular filtration rate ratio may be a more expedient way of improving risk assessment of CIN than the more common practice of estimating CM dose from volume alone [50]. After intravascular administration of CM in rabbits, a urinary concentration higher than 100 mg/mL of iodine has been measured [15]. However, we found that the iodine alone does not cause renal cell apoptosis.

We observed that the cytotoxic effect, although maximum at 3 h, was mostly (≈85%) observed already at 15 min of incubation. This suggests that even a short period of exposure activates the cascade leading to apoptosis and therefore what is being observed at the later time periods represents mostly the cumulative effect of that initial exposure. This finding highlights the importance of strategies limiting the exposure of the kidney to the toxins contained in the contrast agent by generating high urine flow in patients.

The contribution of osmolality to contrast-induced apoptosis is controversial [44,51,52]. Although previous studies demonstrated that the cytotoxicity of high-osmolality contrast media (HOCM) is higher than that of LOCM [44], we did not find any difference in the extent of cell injury between IOCM and LOCM. Furthermore, the cytotoxic effect may be related to CM hypertonicity, since equally hyperosmolal but less hypertonic urea solution failed to induce DNA fragmentation [44]. Factors other than osmolality may contribute to the toxic effect. Ionicity and/or molecular structure (monomeric or dimeric) may be of importance. Heinrich *et al*. [18] demonstrated that at an equal iodine concentration, no significant differences exist between the direct toxic effects of non-ionic monomeric and dimeric CM on renal proximal tubular cells *in vitro*. On the contrary, when comparing the data on a molar basis, the dimeric CM showed a significantly stronger effect on the tubular cells than did the non-ionic monomeric CM. This suggests a greater cytotoxic effect of the dimeric CM molecules. In the last generation of CM (which has a non-ionic, dimeric structure), iso-osmolality has been achieved at the price of an increased viscosity. Indeed viscosity is inversely related to osmolality. High viscous CM compromise renal medullary blood flux, renal medullary erythrocyte concentration, and renal medullary pO_2 [53]. Our *in vitro* experiments allow us to examine the cytotoxic effects of CM on renal cells, eliminating the effects of confounding variables (e.g. hypoxia due to haemodynamic changes or viscosity), which can be found *in vivo*. Therefore, additional studies are necessary to assess whether

molecular structure and/or other components of the CM may induce this cytotoxic effect.

Antioxidant compounds and contrast-induced apoptosis

In the last few years, a number of clinical studies have suggested that NAC may prevent CIN [7,25]. Recently, two additional antioxidant strategies have aroused considerable interest: sodium bicarbonate [27] and ascorbic acid [28]. It has been hypothesized that all these compounds may be effective due to their antioxidant properties.

Our study supports the clinical observation of the effectiveness of NAC and ascorbic acid in preventing contrast-induced apoptosis. This effect is dose-dependent: indeed, the greater the dose, the larger the cellular benefit. This finding supports the clinical observation of the dose-dependency of NAC in preventing CIN [25,54]. The plasma level of NAC ranges from 10 mM (with a dosing regimen of 600 mg/day) to 100 mM (with a dosing regimen of 1200 mg BID) [16,17]. Of note, NAC was more effective against contrast-induced apoptosis than ascorbic acid. In contrast, sodium bicarbonate does not prevent contrast-induced apoptosis. However, recent clinical studies suggest that the sodium bicarbonate seems to be effective in preventing CIN [27]. This discordance may be explained by alternative mechanisms.

We recently demonstrated that the combined prophylactic strategy of sodium bicarbonate plus NAC, but not the combination of ascorbic

acid and NAC, is more effective than NAC alone in preventing CIN. We speculated that NAC and ascorbic acid may work through similar pathways while the protective action of bicarbonate may be different in comparison to NAC and, therefore, additive [26]. The lack of benefit of the combination of NAC and ascorbic acid in preventing contrast-induced apoptosis observed in the present study supports this hypothesis.

Free-radical formation is promoted by an acidic environment typical of distal tubular urine, but is inhibited by the higher pH of normal extracellular fluid [55]. It has been hypothesized that alkalinizing renal tubular fluid with bicarbonate [27] may reduce injury. At physiologic concentrations, bicarbonate scavenges peroxynitrite and other reactive species generated from nitric oxide.[10] In the clinical setting, the higher concentration of HCO_3 in the proximal convoluted tubule may (i) buffer the higher production of H^+ due to cellular hypoxia and (ii) facilitate Na^+ reabsorption through the electrogenic Na/HCO_3 co-transporter [55]. The result of our *in vitro* study does not support the former mechanism. It may be that $NaHCO_3$ may facilitate Na^+ reabsorption: this would mitigate the increase in sodium delivery to the macula densa induced by CM, an effect that results in vasoconstriction of the afferent arteriola through the oricess of tubuloglomerular feedback. Furthermore, in our *in vitro* model, $NAHCO_3$ did not raise the pH of the media in comparison to CM alone.

Conclusion

In conclusion I showed that CM induce apoptosis through the activation of the intrinsic pathway. Pre-treatment with NAC and ascorbic acid but not with sodium bicarbonate prevents apoptosis in a dose-dependent fashion.

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