The role of DNA fragmentation in contrast-induced renal tubular cell damage

A thesis submitted for the degree of Doctor of Medicine in the Faculty of Medicine of the Universität Ulm

by

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Date of dissertation: 05.06.2014
Dedicated to my daughters, Claire and Maria
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Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATCC</td>
<td>American Type Culture Collection</td>
</tr>
<tr>
<td>CARE</td>
<td>cardiac angiography in renally impaired patients</td>
</tr>
<tr>
<td>CM</td>
<td>contrast medium</td>
</tr>
<tr>
<td>CIN</td>
<td>contrast induced nephropathy</td>
</tr>
<tr>
<td>Cf</td>
<td>coefficient</td>
</tr>
<tr>
<td>CT</td>
<td>computertomography</td>
</tr>
<tr>
<td>DNA</td>
<td>desoxyribonuceidacid</td>
</tr>
<tr>
<td>EGF</td>
<td>epidermal groth factor</td>
</tr>
<tr>
<td>GFR</td>
<td>glomeruar filtration rate</td>
</tr>
<tr>
<td>HK</td>
<td>human kidney cell line</td>
</tr>
<tr>
<td>HPV</td>
<td>human papilloma virus</td>
</tr>
<tr>
<td>LMA</td>
<td>low melting agarose</td>
</tr>
<tr>
<td>LOCM</td>
<td>low osmolar contrast medium</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>SCGE</td>
<td>single cell gel electrophoresis</td>
</tr>
<tr>
<td>SD</td>
<td>standard deviation</td>
</tr>
<tr>
<td>yr</td>
<td>year</td>
</tr>
</tbody>
</table>
1. Introduction

1.1 Contrast induced Nephropathy

Contrast mediated clinical examinations are the mainstay of diagnostic and interventional procedures, an integral part of modern medicine. In 2003 the application rate of contrast medium in radiological procedures in the United States approached nearly 80 million, making contrast media the most common drug used to date. [24]. Moreover there is an increasing willingness to examine and treat elderly patients using CT scans [54]. Contrast-induced nephropathy (CIN) resulting in acute renal failure is thus one of the most common scenarios in the clinical setting, leading to high mortality as well as high medical costs universally. The nephrotoxicity of contrast media has been attributed to the osmolality of the compounds, thus prompting evolution of the chemical constitution of contrast media over the years from the primary hyperosmolar compounds to the now commonly used isoosmolar and hypoosmolar compounds.

1.2 Definition of contrast induced acute renal failure

Acute renal failure includes an abrupt and sustained decline in the glomerular filtration rate (GFR) within 48 to 72 hours, an increase in serum creatinine of >0,5 mg per decilitre (44µmol per liter) over the base-line value or an increase of more than 25 % over the base-line value [30, 32, 34, 48, 53, 56]. The frequency of acute renal failure varies significantly depending on the clinical setting. The frequency amongst patients is 1 % at admission, 2 to 5% during hospitalization [22, 46], and as high as 4-15% after cardiopulmonary bypass [26, 56]. The incidence of acute renal failure in Germany with in-patients is 2 to 5% and with patients in intensive care it is even higher with 10 to 30% in the last 4 decades. The lethality has also remained constant in the last 4 decades in the environs of 30% [1,3,4].

Patients with pre-existing renal insufficiency are predisposed to radiocontrast-induced acute renal failure [39]. Patient-related risk factors include pre-existing renal dysfunction, diabetes mellitus, age, congestive heart failure, hypertension,
salt depletion, gender, atherosclerosis, previous history of contrast-induced nephropathy, low effective circulatory volume, myocardial infarction, whereby the combination of pre-existing renal dysfunction with diabetes mellitus and hypertension is seen as very important (table 1) [13]. For diabetic patients, the risk of contrast-induced acute renal failure is directly proportional to the elevation in baseline serum creatinine [36].

Table 1. Risk factors for contrast induced nephropathy. The most relevant patient related and non-patient related risk factors and the relative risk for contrast-induced nephropathy are shown below [13].

<table>
<thead>
<tr>
<th>Risk factor</th>
<th>Odds Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Patient related</strong></td>
<td></td>
</tr>
<tr>
<td>Pre-existing renal dysfunction with elevation in serum creatinine level:</td>
<td></td>
</tr>
<tr>
<td>▪ 1.2-1.9mg/dl (106-176µmol/L)</td>
<td>2.42</td>
</tr>
<tr>
<td>▪ 2.0-2.9mg/dl (177-264µmol/L)</td>
<td>7.37</td>
</tr>
<tr>
<td>▪ &gt;3.0mg/dl (265µmol/L)</td>
<td>12.82</td>
</tr>
<tr>
<td>Diabetes mellitus</td>
<td>5.47</td>
</tr>
<tr>
<td>Age (1-yr increment)</td>
<td>1.02</td>
</tr>
<tr>
<td>Congestive heart failure</td>
<td>1.53</td>
</tr>
<tr>
<td><strong>Hypertension</strong></td>
<td>1.20</td>
</tr>
<tr>
<td><strong>Low effective circulatory volume</strong></td>
<td>1.19</td>
</tr>
<tr>
<td><strong>Myocardial infarction</strong></td>
<td>1.85</td>
</tr>
<tr>
<td><strong>Non-patient related</strong></td>
<td></td>
</tr>
<tr>
<td>Osmolality and content of contrast medium</td>
<td></td>
</tr>
<tr>
<td>In patients with pre-existing renal dysfunction (Low- versus high-osmolality)</td>
<td>0.5</td>
</tr>
<tr>
<td>Volume of contrast medium (per 100ml)</td>
<td>1.21</td>
</tr>
</tbody>
</table>

The administration of low-osmolar or iso-osmolar contrast media has been found to be beneficial in reducing the incidence of CIN among high-risk patients but not among patients without risk factors [1, 4]. The volume of contrast medium administered correlates with the risk of nephropathy [11, 31, 39]. In a series of patients undergoing coronary angiography, each 100ml of contrast medium administered was associated with a significant 12% increase in the risk of nephropathy [39]. It has been established that modulating the volume of contrast medium to the patient's body weight and serum creatinine level minimizes this risk.
Conclusively, the amount of contrast medium used should be kept to a minimum and should not exceed patient-specific maximum doses. The superiority of non-ionic contrast medium as opposed to ionic contrast medium has not been definitely proven. In 2 large studies, the benefit of non-ionic contrast media was limited to patients with pre-existing renal dysfunction [39, 49], whereas a third study showed no benefit of non-ionic over ionic contrast agents in patients either with or without pre-existing renal dysfunction [43].

1.3 Pathogenesis

Nephrotoxins induce acute renal failure through vascular and tubular effects. When vascular injury predominates, the decrease in renal blood flow and increase in glomerular filtration coefficient (Kf) [increased tubuloglomerular feedback] jointly diminish the filtration pressure resulting in the retention of normally filtered blood components. When tubular injury predominates, this leads to reduction of transepithelial resistance, inulin permeability and polarised cellular enzyme release [23, 33]. Membrane permeability increases or cell integrity is compromised resulting in leakage of filtration markers (Tamm-Horsfall proteinuria), nephron obstruction and subsequently decreased intraluminal fluid volume. Intraluminal obstruction results in an increase in the capillary pressure to sustain glomerular filtration. Clinically this compromises nephron integrity, causing a reduction in glomerular filtration rate and a rise in serum creatinine [41].

Vascular ischemia is a major contributor to contrast-induced acute renal failure given that acute changes in renal hemodynamics have been reported in various experimental protocols. In muscle beds, acute vasoconstriction is followed by prolonged vasodilatation. In contrast, the renal circulatory response is initially a transient vasodilatation followed by prolonged vasoconstriction. A further redistribution of blood flow resulting in the reduced flow to the medulla is apparent within the kidney [13, 35]. In experiments in rats, a severe morphologic injury, which correlated with reduced creatinine clearance, was detected in the outer medulla, an area prone to ischemic injury in the thick ascending limb segments. The ischemic injury was therefore seen to be a result of a disparity in metabolic supply and demand due to vasoconstriction concurrent with increased metabolic
activity. The increase in metabolic activity is a consequence of increased sodium delivery [18, 35].

After exposure to contrast media, renal tubular cell injury is accompanied by significant decreases in tubule potassium, adenosine triphosphate, total adenosine nucleotide, and basal uncoupled respiratory rates as well as a significant increase in tubule Ca\(^{2+}\) content. Contrast agents were found to reduce the activity of the antioxidant enzymes catalase and peroxide dismutase in the renal cortex of volume-depleted rats, subsequently suggesting oxidant-mediated injury as a mechanism of cytotoxic effect in the pathogenesis of contrast induced renal failure [22].

Fragmentation of DNA, the hall mark of apoptosis, and other morphological characteristics of programmed cell death have been documented in renal tubular, glomerular, vascular endothelial and smooth muscle cells of the heart and kidneys in the rat model of contrast-induced nephropathy [5, 55].

1.4 Development of contrast medium

Iodinated contrast media are water-soluble substances, which are administered either intravenously or directly applied into the organ of interest. 90% of contrast medium (CM) is eliminated via the kidney. Contrast media differ in their carrier molecule, the number of iodine molecules per bonded carrier molecule [32, 34, 48, 51] and in their physico-chemical characteristics, i.e. osmolality, viscosity and hydrophilia. The radioopacity of CM is dependent on the iodine concentration; osmolality influences compatibility and viscosity influences fluidity.
In 1923, E.D. Osborne, a 28-year-old venereologist at the Mayo clinic, noticed that syphilis patients who had been treated with sodium iodine had urine that was opaque to x-ray examination. In 1933, Moses Swick proposed the use of iodinated benzene compounds as contrast agents.

Early contrast agents were ionic monomers (Figure 1a) with an ionizing carboxyl group attached to the first carbon of the iodine-containing benzene ring, which forms the basic structure of all contrast agents [44]. These agents had a high osmolarity (1500 to 1800 mosmol/l) and were extremely hyperosmolar relative to plasma. The osmolality of contrast agents is largely related to the size of the molecule of contrast agent and the number of particles in solution [44].

The strategies for lowering osmolality include; making the agents nonionic, thereby reducing the number of osmotic particles; maintaining the ionic character but doubling the number of iodine atoms per anion which has an identical effect.

By the late 60’s the idea of reducing the osmolality, thereby reducing the toxicity of contrast agents via formulation of a non-dissociating contrast agent with subsequently less particles in solution had taken root.

Metrizamide, the first-generation of non-ionic contrast media had a substantially lower osmolality but was unstable in solution and too expensive. The second-generation monomers such as Iohexol (Figure 1B) were much more stable but still hyperosmolar relative to plasma with 500-850mOsm per kilogram.
The third-generation contrast agents are dimers, linking two molecules of contrast agent together through a common side chain and thereby increasing the size of the molecule in solution and further reducing the osmolality. Iodixanol (Figure 1C) is the first third-generation dimeric contrast agent and is iso-osmolar. It also induces less pain when injected into small-caliber arteries. The dimeric structure, however, increases the viscosity, rendering the contrast material more difficult to inject [44].

1.5 Problem definition

The new iso-osmolar contrast agent Iodixanol was assumed to be less nephrotoxic than low osmolar contrast agents commonly used. The aim of this study was to compare the difference in the risk of acute renal failure after administration of either iso-osmolar Iodixanol or low-osmolar Iopromide and secondly to decipher the relationship between dosage and duration of exposure and the degree of cell destruction after administration. For that purpose we used a cell culture based in-vitro assay and compared the potential of Iodixanol and Iopromide to induce DNA-damage in the well-established human kidney cell-line HK-2.
2. Materials and Methods

2.1 Research materials

The experiments were performed in the laboratory of the nephrology division of the university of Ulm.

2.1.1 Laboratory equipments

All laboratory equipments used in this study are tabulated as follows

Table 2. Source of laboratory equipments used in this study.

<table>
<thead>
<tr>
<th>Article</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Microscopic slides with frosted borders 76 x 26 mm</td>
<td>Menzel Glaser</td>
</tr>
<tr>
<td>Cover slips 24 x 60 mm</td>
<td>Menzel Glaser</td>
</tr>
<tr>
<td>Eppendorf pipettes with tip cones / Eppendorf aliquots</td>
<td>Sigma (München)</td>
</tr>
<tr>
<td>Gel electrophoresis chamber with 14 cm electrode interval</td>
<td>Biorad</td>
</tr>
<tr>
<td>Power supply unit</td>
<td>Biometra</td>
</tr>
<tr>
<td>Fluorescence microscope</td>
<td>Zeiss (Oberkochen)</td>
</tr>
<tr>
<td>Digitalkamera Canon Powershot G2</td>
<td>Canon Corp</td>
</tr>
<tr>
<td>Image analysis-Software Scion Image Beta 4.0.2</td>
<td>Scion Corp [17]</td>
</tr>
<tr>
<td>Comet-Assay' Macro</td>
<td></td>
</tr>
<tr>
<td>Incubator</td>
<td>Heraeus, Germany</td>
</tr>
<tr>
<td>Tissue culture plates (6 well)</td>
<td>Falkon, Becton</td>
</tr>
<tr>
<td></td>
<td>Dickinson Labware, Franklin Lakes, NJ,</td>
</tr>
</tbody>
</table>
2.1.2 Chemicals

All chemicals possessed analytical purity grade and were procured from the following companies:

Table 3. Source of all chemicals used in this study.

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Agarose</td>
<td>SeaKem (Rochland, USA)</td>
</tr>
<tr>
<td>low melting-Agarose</td>
<td>(LMA)</td>
</tr>
<tr>
<td>Biozym</td>
<td>(Hess. Oldendorf)</td>
</tr>
<tr>
<td>Bovine Serum-Albumin (BSA)</td>
<td>Sigma (München)</td>
</tr>
<tr>
<td>Dimethylsulfoxid (DMSO)</td>
<td>Sigma (München)</td>
</tr>
<tr>
<td>ddH₂O</td>
<td>Pharmacy of the Universitätsklinikums (Ulm)</td>
</tr>
<tr>
<td>Ethylenediamintetraacetat (EDTA)</td>
<td>Merck (Darmstadt)</td>
</tr>
<tr>
<td>Ethanol 99%-ig</td>
<td>Pharmacy of the Universitätsklinikums (Ulm)</td>
</tr>
<tr>
<td>Ethidiumbromid</td>
<td>Roth (Karlsruhe)</td>
</tr>
<tr>
<td>Glycerol 99%-ig</td>
<td>Pharmacy of the Universitätsklinikums (Ulm)</td>
</tr>
<tr>
<td>Ethansulfonsäure (HEPES)</td>
<td>Sigma (München)</td>
</tr>
<tr>
<td>HCl (konzentriert)</td>
<td>Merck (Darmstadt)</td>
</tr>
<tr>
<td>NaCl</td>
<td>Merck (Darmstadt)</td>
</tr>
<tr>
<td>NaOH</td>
<td>Merck (Darmstadt)</td>
</tr>
<tr>
<td>KCl</td>
<td>Merck (Darmstadt)</td>
</tr>
<tr>
<td>KOH</td>
<td>Fluka - Buchs (CH)</td>
</tr>
<tr>
<td>Phosphat buffered saline (PBS)</td>
<td>Merck (Darmstadt)</td>
</tr>
<tr>
<td>Tris-Puffer</td>
<td>Merck (Darmstadt)</td>
</tr>
<tr>
<td>Triton X-100</td>
<td>USB - Ohio (USA)</td>
</tr>
<tr>
<td>0.15% Trypsin</td>
<td>Sigma (München)</td>
</tr>
<tr>
<td>Culture medium</td>
<td>Sigma (München)</td>
</tr>
</tbody>
</table>
2.1.3 Solutions

Lysis stock solution (Contents in 1000ml)
- 2.5 M NaCl
- 100 mM EDTA
- 10 mM Tris-Buffer
Graduated with NaOH to a pH of 10, filled with ddH₂O to 1000 ml and stored at room temperature 89 ml of the base solution was mixed with 1 ml Triton X-100 und 10 ml DMSO before use. This solution was stored at 4° C for 60 minutes before usage.

Elektrophoresis buffer (pH >13):
- 300 mM NaOH / 1 mM EDTA
base solution:
- 10 N NaOH
- 200 mM EDTA
Neutralisationsbuffer:
- 0.4 M Tris-Puffer
- filled to 1000 ml with ddH₂ O
- graduated to a pH- value of 7.5  with HCl
- stored at room temperature

LMA-Aliquots
0.5 % of low melting Agarose was prepared from 100 mg Agarose and 20 ml PBS and heated till the Agarose dissolved. The low melting Agarose vials were placed in a 37° C water bath to cool.

Staining solution
Ethidiumbromid (10-fold concentrated base solution: 200µg/ml)
- 10 mg Ethidiumbromid dissolved in 50 ml ddH₂O
- 1-fold concentrated staining solution 20µg/ml
2.2 Cell culture

2.2.1 HK-2 cell line

Human kidney 2 (HK-2) cells utilised in this experiment were obtained from the proximal tubule in the Cortex of a healthy human kidney and the ATCC (American Type Culture Collection, Manassas USA) used these cells to generate a stable cell line. The cells were immortalised through transformation with genes of the Human-Papilloma-Virus 16 (HPV-16) E6/E7 since this does not cause any significant change in the phenotyp or cell function. The thus cloned HK-2 cells were cultivated for over a year as a monolayer in keratonocytes (serum-free medium). The cell growth is EGF-dependent. The E6/E7 gene could be isolated in the genom of HK-2 cells via PCR (polymerase chain reaction). HK-2 cells exhibit morphological and functional characteristics unique to the cells of humane tubulus epithel which include long and closely aligned microvilli, junctional complexes as well as enzymes quintessential to the ciliated border such as alcaline phosphatase, acid phosphatase, glutamytransferase and leucin-aminopeptidase. HK-2 cells are recognised by antibodies against cytokeratin, vimentin, fibronectin and 3,1-integrin but go undetected by Factor VIII, CALLA-Endopeptidase and 6.19 antigen. This is additional evidence of its tubular derivation and of the spezific cell phenotype. HK-2 cells regulate a chlorid-dependent phlorizin-sensitive glucose transport as well as the stimulaton of adenylatcyclase through parathyroid hormone, however not through antidiuretic hormone, additional characteristics specific to cells of the proximal tubulusepithel.

HK-2 cells possess the ability to perform gluconeogenesis as well as formation and storage of glycogen. In experiments of cell damage through $\text{H}_2\text{O}_2$ the results of newly isolated proximal tubulus segments could be reproduced via HK-2 cells. The suitability of HK-2 cells in studies of cell damage and repair mechanisms could thus be elucidated [15, 42].
2.2.2 Medium and materials for cell culture

- Culture medium: DMEM/HAM’S F-12 (1:1) = 0.648 g N-Acetyl-L-Alanyl-L-Glutamin/l, pH 7.4 (Biochrom KG, Berlin)
- Foetal bovine serum = FCS (Biochrom KG, Berlin)
- Viralex Trypsin/EDTA (1x) = 0.5 g Trypsin/l, 0.2g EDTA/l, 0.85g NaCl/l, w/o Ca²⁺/Mg²⁺ (PAA Laboratories, Linz, Austria).
- PBS DULBECCO’S = 150 mM NaCl, 2.7 mM KCl, 6.5 mM Na₂HPO₄, 1.5 mM KH₂PO₄ (Gibco, Karlsruhe).
- Tissue culture cylinders with 250 ml filter (Greiner Labortechnik, Frickenhausen)
- Petri dishes: Primaria Tissue Culture Dish, 100 x 20 mm (Becton Dickinson Labware, Franklin Lakes, NJ, US)

2.2.3 Contrast media

- Iopromide (Ultravist 300 ®) 1ml contains: 0.632g iopromide, Natriumcalciumedetat, Trometamol, hydrochloric acid, injection water, Iodine content 300 mg/ml (Schering Deutschland GmbH, Berlin).
Iodixanol (Visipaque 270) 1 ml contains: 550 mg iodinaxol, Natriumcalciumedetat, Trometamol, Calciumchlorid, Natriumchlorid, hydrochloric acid, injection water, iodine content 270 mg/ml (Amersham Buchler GmbH & CO.KG, Braunschweig)

2.3 Cell culture assay and evaluation of cell damage

2.3.1 Detection of DNA damage using the comet assay.

Figure 3: From cell culture to DNA-fragmentation; an overview of the comet assay [57].

The Comet Assay or single cell gel electrophoresis (SCGE) assay is a rapid, sensitive and relatively simple method for detecting DNA damage at the level of individual cells [50]. It combines the simplicity of biochemical techniques for detecting DNA single strand breaks (strand breaks and incomplete excision repair sites), alkali-labile sites, and cross-linking, with the single cell approach typical of cytogenetic assays. This assay was first introduced by Ostling and Johanson in 1984. Theirs was a neutral assay in which the lysis and electrophoresis were done under neutral conditions. Staining was done with acridine orange. The image obtained looked like a “comet” with a distinct head, comprising of intact DNA and a tail, consisting of damaged or broken pieces of DNA hence the name “Comet” Assay. The approach of Ostling and Johanson was based on previous work published by P Cook in 1976, who developed a method for investigating nuclear structure based on the high salt lysis of cells in the presence of non-ionic
detergents [47].

Using this technique, a small number of cells suspended in a thin layer of Agarose on a slide is lysed, electrophoresed and stained with a fluorescent DNA binding dye. Cells with increased DNA damage demonstrate an increased migration of chromosomal DNA from the nucleus towards the anode in the shape of a comet. In the alkaline comet assay DNA single strand breaks, double strand breaks as well as alkali-labile sites cause DNA migration [50].

2.3.2 Preparation of slides

Slides with frosted borders were cleaned with ethanol and labelled with a solvent resistant marker. 20 ml PBS was added to 300mg of normal melting Agarose yielding approximately 1.5% of normal melting Agarose and brought twice to boil. The slides were submerged up to the frosted borders into the hot Agarose to ensure proper adhesion and left to dry.

2.3.3 Cell preparation and inoculation with iodixanol and iopromide

Monolayer cell cultures in 6-Well plates were inoculated with 4 concentrations of iodixanol and iopromide respectively. One cell layer, which would later serve as the control cell line, was not inoculated with contrast media. To terminate contrast activity contrast was siphoned off the 6-Well plates in accordance with a designated time of action ranging between 10 minutes and 2 hours and the cells were further neutralised using PBS. To separate the cells from the monolayer each well was inoculated with 500μl 0.15% trypsin for 30 seconds and subsequently placed in an incubator for 5 minutes. The well contents were then transferred into Eppendorf pipettes and 1ml PBS was added to each pipette. All pipettes with cell content were centrifuged at 2000x in 10 minutes, the liquid suspensions were drained and the cell residue subsequently mixed with 120 μl low melting Agarose then transferred to a slide. Cover slips were placed on the slides and the slides were transferred to a tray. To avoid direct light irradiation and thus prevent additional DNA damage, the trays were sealed with aluminium foil. The trays were placed into a refrigerator for
approximately 2 minutes. The cover slips were discarded and the slides submerged into cold, freshly prepared lysing solution.

2.3.4 Electrophoresis and staining

The electrophoresis was performed in an ice bath (4°C) with exclusion of light using aluminium foil.

After 1 hour the slides were removed from the lysing solution and placed in the electrophoresis chamber on the horizontal gel box at the anode (+) end. The buffer reservoirs were filled with freshly made electrophoresis buffer until the slides were completely submerged in fluid.

To permit unwinding of the DNA and expression of alkali-labile damage the cells were left in the alkaline buffer for 20 minutes. Electrophoresis was performed at 25V and 300mA for 30 minutes. The slides were removed from the electrophoresis chamber, covered with neutralisation buffer 3 times for about 5 minutes respectively and left to dry.

The slides were then covered for 5-10 minutes with ethanol and left to dry at room temperature. Staining of the slides was performed with 50 µl ethidium bromide solution per slide.

2.3.5 Evaluation of DNA effects

To visualize DNA damage observations were made of ethidium bromide-stained slides at 400x magnification using a fluorescence microscope equipped with an excitation filter of 515-560 nm and a barrier filter of 590 nm and linked to a gated CCD Camera (Figure 4). The thus obtained images were converted from their acquisition format (JPEG) into TIFF-images in order to facilitate their subsequent evaluation via a PC-based image analysis system (Scion image, Scion Corporation, Version 4. 0. 2 Beta). This system was complemented with a macro to strengthen its ability to assess the parameters of DNA migration relevant to comet assay [17]. Broken DNA migrates farther in the electric field; cells with DNA fragments thus resemble a comet with a fluorescent head and a tail region, which increases proportionally to cell damage.

The cells were then analysed in groups of 50 randomly selected cells, whereby extensively damaged cells were excluded.
Tail moment

A. Control

B. Iopromide

Figure 4: Microscopic image of HK-2 cells in this experiment, A: undamaged, B: damaged with migrated DNA fragments as comet.

2.3.6 Contrast agent concentrations

The monolayer cell cultures in the 6-wells were treated with 3 different concentrations of both iodixanol and iopromide, namely with 20 mg (74 µl), 80 mg (296 µl) and 120 mg (444 µl) for iodixanol as well as 20 mg (66.7 µl), 80 mg (266.7 µl) and 120 mg (480 µl) for iopromide respectively. The time of action for each concentration was limited to 10 minutes, 30 minutes, 1 hour and 2 hours, giving rise to a total of 12 experiments for both iodixanol and iopromide respectively. Data from a total of 600 cells was obtained and analysed for each contrast medium and for each experiment. Altogether more than 40 test runs were performed.

2.4 Statistics

In this analysis the statistical variants used were tail length and tail moment. The tail moment (tail length x DNA percentage in the tail) was used to define the degree of DNA migration and thus to quantify DNA damage.
The Utilization of tail moment as parameter is advantageous in that it combines the tail length and its DNA content and thus renders a more accurate illustration of DNA damage compared to the other parameters respectively [17, 27].

The tail moment was illustrated as the mean ± standard deviation (SD). The Student’s t-test was used to test for statistically significant differences between two mean values. Statistically significant differences between the two contrast agents were assessed with two-way analysis of variance (ANOVA; GraphPad Prism-Version 4.0, GraphPad Software, San Diego, California). Differences were considered significant if p < 0.05 and highly significant if p < 0.01. Data are expressed as pooled mean of the 50 comet assays each scored per slide for each time point and for each concentration on 3 different days.
3. Results

In order to investigate whether iodixanol and iopromide induce DNA damage in human renal tubular cells, HK-2 cells were incubated with increasing doses of iodixanol and iopromide at different time points (10, 30, 60 and 120 minutes) and analyzed by comet assay.

Oxidative stress was defined as characteristic DNA strand breaks. Iopromide as well as iodixanol induced concentration- and time-dependent DNA fragmentations in HK-2 cells.

In Figure 5, the amount of DNA damage in contrast media treated cells was different from the low levels in control cells, but a significant effect was only observed at a concentration of 120 mg iodine /ml for both contrast media (p<0.001). The effect at high concentrations of contrast media was also significant compared with the lower concentrations of both contrast media.
**Figure 5:** Concentration-dependent DNA-fragmentation induced by iopromide and iodixanol in the comet assay. Hk-2 cells were incubated with control medium (DMEM) or medium containing 20, 80 or 120 mg iodine/ml of contrast medium. The untreated cells are compared with cells treated with 120mg of both media. A; 10 minutes of incubation time, B; 30 minutes of incubation time, C; 1 hour of incubation time, D; 2 hours of incubation time. Results are expressed as Mean ± SD of three independent experiments each (n= 50).

Figure 5 depicts DNA fragmentation induced by iopromide and iodixanol, comparing untreated cells (control experiment) with cells treated with 120mg of both media. There is a statistical significant increase in tail moment in the cells treated with 120mg of both media compared to the untreated cells (control).

**Figure 6:** Concentration-dependent DNA fragmentation induced by iopromide and iodixanol in the comet assay. HK-2 cells were incubated with control medium (DMEM) or medium containing 20, 80 or 120 mg iodine per ml. The cells in the control experiment are compared with the cells treated with 20mg, 80mg and 120mg of each medium. A: 10 minutes of incubation time, B: 30 minutes of incubation time, C: 1 hour of incubation time, D: 2 hours of incubation time. Results are expressed as pooled mean ± SD of three independent experiments with n = 50 each (* p< 0.001 versus control).
Figure 6 outlines the DNA-damaging effect of increasing concentrations (20 mg, 80 mg and 120 mg iodine per ml) of both contrast media after an incubation period of 10 minutes, 30 minutes, 1 hour and 2 hours respectively. Effects were compared to that on untreated cells, the control experiment. The amount of DNA damage in contrast media treated cells was different from the low levels in control cells, and a highly significant effect was observed ($p < 0.001$) as early as 10 minutes for both contrast media with the concentrations of 80 mg and 120 mg iodine per ml (Figure 6 A). Significant differences were seen at 1 hour for both contrast media and all concentrations (Figure 6 C), as well as for 2 hours incubation time (Figure 6 D).

![Figure 7: Comparison between iopromide and iodixanol after 1 (A) and 2 (B) hours of incubation time. Pooled mean ± SD, n = 50 each on 3 different days; two-way ANOVA showed a statistically significant difference between two contrast media: $p = 0.039$ for the 1 hour (A), and $p = 0.025$ for the 2 hour incubation time (B), respectively.](image)

To investigate the difference between DNA damage induced by both contrast media a two-way ANOVA was performed. The results are depicted in Figure 7. A statistically significant difference was found between the two contrast media at 1 hour and all concentrations (Figure 7A). The difference was also statistically significant with an incubation period of 2 hours (Figure 7B).
4. Discussion

Evidence suggests that reactive oxygen species (ROS) may be involved in contrast media-induced nephrotoxicity, since it has been found that the levels of $O_2^-$, $H_2O_2$ and HO$^-$ increase with contrast media treatment [19, 34]. It has furthermore been suggested that direct toxicity of contrast molecules may affect renal tubular cells, causing damage through reactive oxygen species [2, 19, 37]. The aim of this study was to explore and to compare the degree of nephrotoxicity of the low-osmolar iopromide and the iso-osmolar iodixanol on proximal renal tubules cells (HK-2) using DNA-fragmentation as a marker for apoptosis and consequently oxidative stress. Exposure time and dose range of the respective contrast media were selected as variables.

The dose range of CM chosen for these studies was carefully determined to allow for clinical applicability. CM are typically administered at a dose of 1.5 ml/kg, resulting in plasma iodine concentrations of 10 mg iodine /ml. Higher doses, however are not uncommon, leading to plasma iodine concentrations of 15-20 mg iodine /ml [14]. Consequently concentrations in the proximal tubule will be considerably higher as 60-80% of the water and solute content of glomerular filtrate is reabsorbed in this portion of the renal tubule [55]. We therefore chose a range of contrast concentrations that would potentially include patients with normal and compromised renal function (20-120 mg I/ml).

We demonstrated that the oxidative damage of HK-2 cells is an early event following the application of contrast media [29]. This may be as a result of an up-regulation of the primary defense mechanism; the antioxidative system and/or complementary DNA repair system. Furthermore, the induction of oxidative DNA-fragmentation by both classes of contrast media in vitro was time and concentration dependent. The incubation times and iodine concentrations used in the current experiments may be pathophysiologically relevant, since they may occur in the clinical setting. Induction of DNA fragmentation was observed even at the lowest iodine concentration of 20 mg/ml, which represents a very low dose of CM. Cell damage was also observed as early as 10 minutes after application of CM.

In our experiments time does appear to enhance the injury inflicted by contrast media. This corresponds with clinical findings in a scenario with impaired renal function. At all concentrations, both media showed a significant amount of DNA
fragmentation as early as after 30 minutes of incubation, compared to the non-treated cells. (Figure 6A-D). Significant difference between both media was observed after 1 and 2 hours of incubation time, with iodixanol causing more DNA damage (Figure 7). This effect has also been observed in patients in whom the iso-osmolar iodixanol induced kidney dysfunction [34]. Reduced nephron number is associated with a higher and longer lasting contrast agent load in the single tubule. The half-life of contrast media in patients with normal kidney function is about 2 hours whereas patients with kidney failure may have the half-life extended to as much as 70 hours [28]. These results could explain, why mainly kidneys with impaired function show contrast medium induced nephrotoxicity.

In their Meta-analysis, Tepel et al. concluded that significant differences have been identified between contrast agents due to their physicochemical properties, and low-osmolar or iso-osmolar contrast media should be used to prevent CIN in at-risk patients [52].

Although the composition of contrast media has undergone profound changes in the past 5 decades with a subsequent reduction in the rate of adverse side effects, there seems to be no clear paradigm as to whether iso-osmolar non-ionic contrast media possess a lower nephrotoxicity compared to low osmolar nonionic contrast media.

The Cardiac Angiography in Renally Impaired Patients (CARE) trial randomized 414 patients at high risk for contrast-induced acute renal failure to angiography with either iopamidol or iodixanol, finding no difference in any measure of acute renal failure between these two groups [49]. A Meta-analysis of these trials [16] advocates equivalent safety of iso-osmolar and low-osmolar contrast, with the possible exceptions of ioxaglate and iohexol. The evidence of our study is at odds with this conclusion. In another Meta-analysis REED et al., suggested that iodixanol, when compared with LOCM (low osmolar contrast medium) overall, is not associated with less contrast-induced acute renal failure. The relative renal safety of LOCM compared with iodixanol may vary based on the specific type of LOCM [38].

Beeri et al. found that in isolated perfused kidneys, DNA breaks were present in medullary tubules as early as after 10 minutes of local hypoxia and were prevented by reduction of metabolic work. [5]. In a model of radiocontrast-induced acute renal failure, DNA breaks were detected selectively along thick ascending
limbs as early as 15 minutes following insult, preceding overt morphological
damage. They thus concluded that hypoxia induces rapid DNA fragmentation
along thick ascending limbs, where programmed cell death could play an
important role in nephron injury and kidney failure. [5].

Seeliger et al found that iopromide markedly increased urine production whereas
iodixanol, which caused less diuresis, significantly enhanced urine viscosity. Only
high-viscosity agents such as iodixanol decreased renal medullary bloodflow,
erthrocyte concentration, and pO₂. They also found that iodixanol prolonged the
tubuloglomerular feedback response and increased plasma creatinine levels to a
greater extent than iopromide [45]. This is in line with the findings in our study
[28]. On the other hand, they concluded that the viscosity of contrast media might
play a significant role in contrast-induced nephropathy. This notion though seems
unlikely in our study as the utilization of an in vitro cell system negates the effect of
viscosity for iodixanol, thus proving the nephrotoxic effect of iodixanol independent
of the effect of viscosity. Furthermore, the cytotoxic effect does not seem to be
caused by iodine or osmolarity <830mOsmol/L [40].

Renal cell damage was observed for both contrast media only at high
concentrations as presumably occurs in patients with impaired renal function. Due
to the fact that the effect was observed in both contrast media, the idea that iso-
osmolar contrast media are less nephrotoxic than low-osmolar contrast media is
unlikely. This has been a controversial issue to date [1, 3, 6].

The clinical relevance of this study is subject to the limitations of an in vitro study
with the lack of decreasing contrast concentrations in and absence of a continuous
blood supply to tubules. In addition, other sources of error may include an
inaccuracy in the administration of the contrast medium, as well as undue
exposure to light during test runs.
5. Summary

Background
Intravascular administration of iodinated contrast media continues to be a common cause of hospital-acquired acute kidney injury. Accumulating evidence suggests that radiocontrast agent induced nephrotoxicity is associated with increased oxidative stress, which leads to renal tissue damage with DNA fragmentation. We therefore tested whether an iso-osmolar contrast medium (Iodixanol) causes less oxidative DNA damage to renal tubular cells compared to a low-osmolar contrast medium (Iopromide).

Method
In our study we incubated human kidney cells (HK-2 cells) in a predefined period of time (10 minutes-2 hours) with increasing concentrations of Iodixanol and Iopromide (20-120mg/ml iodine) and compared the amount of DNA damage induced on the cells. DNA damage was analysed using a single gel electrophoresis technique otherwise known as the comet assay.

Results
The findings show that the two different classes of contrast media induce DNA fragmentation in vitro in a time and concentration dependent manner. DNA fragmentation was maximal at 2 hours with 120 mg for iopromide and iodixanol; both were significantly different from the control value (Student’s t-test; p < 0.001). After 1 and after 2 hours and for all concentrations, iodixanol produced significantly higher DNA fragmentation than iopromide (ANOVA for 1 hour p = 0.039 and 2 hours p = 0.025, respectively).

Conclusion
On the basis of the paradigm that DNA strand breaks or fragmentation are synonymous to oxidative stress, we effectively demonstrated that contrast media induced oxidative stress in HK-2 tubule cells. Renal cell damage was observed for both contrast media at high concentrations such as is presumed to occur in patients with impaired renal function; this effect is much more evident in iodixanol than it is in iopromide. Collectively, our results demonstrate that an iso-osmolar contrast medium induced greater oxidative stress and consecutive DNA damage than a low osmolar agent in HK-2 cells. This is contrary to the widespread notion
that iso-osmolar contrast media may be less nephrotoxic than low-osmolar contrast media and could provide an explanation for the nephrotoxicity observed with iodixanol in clinical practice.
6. References


12. GICHNER T., PATKOVÁ Z., SZÁKOVÁ J. & DEMNEROVÁ K. 2006. Toxicity and
DNA damage in tobacco and potato plants growing on soil polluted with heavy metals. *Ecotoxicology and environmental safety* 65: 420-426.


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8. Curriculum vitae

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