Nephrotoxic effects of iodinated x-ray contrast media *in vitro* and *in vivo*:
Effects on cultured NRK 52-E cells, development of an animal model of impaired renal function and evaluation of biomarkers of nephrotoxicity

Master's thesis

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June 2007

Preface

This master's thesis in toxicology is a part of a master's degree in biology at the University of Oslo. The work on this thesis was carried out at GE Healthcare, Preclinical Sciences (PCS) - Biology, from November 2005 to June 2007. The work was supervised by senior research scientist Oddvar Myhre (GE Healthcare). Steinar Øvrebø was the internal supervisor from the University of Oslo.

Within hospitals, medicine personal, and radiographer the problems related to administration of iodinated x-ray contrast media (ICM) are well known. However, for the convenience of new readers, the introduction in this thesis has aimed to give a brief and general introduction to ICM. Furthermore, appendix 1 lists and classifies all the contrast agents mentioned throughout this thesis. The background chapter in this master's thesis is more directly related to the aims of the study.

As part of this thesis an *in vitro* lab was organized and established at the PCS Biology. Due to the time constraint of this master's thesis, and the complexity of some of the methods, assistance was given in some of the methods. The chemical analysis inductively coupled plasma atomic emission spectrometry (ICP-AES) was carried out with assistance from chemists at GE Healthcare. Histopathology processing, and the clinical chemistry analyses were performed at PCS Biology laboratories. Cytospin preparation of the urine sediments was performed at the Veterinarian Institute (Oslo). Furthermore, all histopathological and cytological evaluation was performed by a veterinarian pathologist.

Acknowledgements

First of all, I want to thank my supervisor senior research scientist Oddvar Myhre for good ideas, valuable discussions, and all the help during the time course of this master's thesis.
I would also like to thank senior research scientist Tore Skotland and Derek Grant for your help in the process of writing.

A special thanks to Ph.D. Richard W. Doughty for your expertise and help in histology and cytology - it was very much appreciated. Thanks to all those who has assisted the work in this thesis: Ivar Martinsen and Grete A. Friisk for ICP-AES assistance; Ann S. Langøy for histopathology processing; Brit H. Engebretsen for clinical chemistry analyses; Stein Waagene, Merete Høyem and Richard W. Doughty for assistance in *in vivo* studies. I also want to thank the rest of the PCS - Biology staff at GE Healthcare for your kindness to help me every time I ask.

I want to thank my friends, especially Stine, Kikki, and Paula for taking interest in my work, and for your encouragement. Thanks to my parents for your endless support, and always believing in me. Finally, a special thanks to my dear Martin for your encouragement throughout the entire process, and for putting up with me during the process of writing.

Hanne Jensen

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Abstract

Iodinated x-ray contrast media (ICM) are used to improve the visibility of internal structures of the body in an x-ray image due to their ability to attenuate x-rays, providing enhanced contrast between regions of interest and the surrounding tissues. Unfortunately, administration of ICM is associated with adverse side effects, including contrast-induced nephropathy (CIN) which remains one of the most important complications of ICM. The work reported in master's thesis was aimed at investigating the nephrotoxic effects of ICM *in vitro* and *in vivo*. This was done by evaluating the effects of ICM in a rat kidney cell line (NRK 52-E), by developing an animal model of impaired renal function to study *in vivo* effects of ICM, and evaluating potential biomarkers of renal damage caused by ICM in the developed model.

Three methods were selected to evaluate the toxicity of three low-osmolal and one iso-osmolal ICM in the NRK 52-E cells, comprising the trypan blue exclusion assay, the MTT assay, and cell death assessment on formalin fixed cells. Cell morphology was included to supplement the selected tests, and an analytical method, ICP-AES, was used to measure uptake of ICM in the cells. An impaired renal failure model to test ICM in rats was developed by pre-tretment with gentamicin. Creatinine, cystatin C, N-acetyl-β-D-glucosaminidase (NAG), γ-glutamyl transferase (GGT), alpha glutathione-S-transferase (α-GST), total protein, and urine cytology were tested as biomarkers of renal damage caused by ICM in the rat-gentamicin model.

The osmolality of the contrast agents appeared to be a major cause for the observed *in vitro* toxicity. A marked difference in cell death was observed between low-osmolal and iso-osmolal ICM, with iso-osmolal being the least toxic. However, the different chemical structures of low-osmolal ICM may also contribute to the toxicity. In the present work uptake of ICM was most likely due to fluid phase endocytosis, and as a consequence, reduced cell viability. The rat-gentamicin model of impaired renal damage proved to be promising and could be used to compare toxicity of different classes of ICM, and for screening novel candidates. Of the biomarkers evaluated, GGT and total protein proved to be suitable biomarkers of renal damage caused by ICM, whereas NAG, α-GST and urine cytology were regarded as promising biomarkers, but which need further evaluation.

Abbreviations

α-GST	α-glutathione S-transferase
ANOVA	analysis of variance
ARF	acute renal failure
BCA	bicinchonic acid
bcl-2	B-cell lymphoma 2
bw	body weight
CDI	cell death index
CIN	contrast-induced nephropathy
EDTA	ethylenediaminetetraacetate
eGFR	estimated glomerular filtration rate
EIA	emzyme immuno assay
ELISA	enzyme linked immuno sorbent assay
GFR	glomerular filtration rate
GGT	γ-glutamyl transferase (also known as γ-glutamyl transpeptidiase)
GSH	reduced glutathione
GST	glutathione transferases
HOCM	high-osmolal contrast medium or high-osmolal contrast media
HPF	high power field
i.m.	intra muscular
i.v.	intravenous
ICM	iodinated contrast medium or iodinated contrast media
ICP-AES	inductively coupled plasma atomic emission spectrometry
IOCM	iso-osmolal contrast medium or iso-osmolal contrast media
IU	international units
LOCM	low-osmolal contrast medium or low-osmolal contrast media
mOsm	milliosmole
NAG	N-acetyl-β-D-glucosaminidase
NSAIDS	nonsteroidal anti-inflammatory drugs
p	probability
PBS	phosphate buffered saline

ROS	reactive oxygen species
rpm	rotations per minute
s.c.	subcutaneous
SD	standard deviation
SEM	standard error of mean

1. Introduction

Ever since the discovery of x-rays by Röentgen in 1895, several attempts have been made to increase the diagnostic yield. X-rays are a form of electromagnetic radiation with a wavelength in the range of 10 to 0.01 nm. The extent of x-ray attenuation differs for bones, muscles, fat and other tissues in the body. By using a special photographic plate it is possible to take pictures of organs and bones for diagnostic and therapeutic purposes. Soft tissue and vessels absorb the least x-rays, and hence it is not possible to take pictures of good quality by using only x-rays. The key feature of iodinated x-ray contrast media (ICM) is the attenuation of x-rays within the diagnostic range. The areas of the body in which the ICM are localized will appear white on the x-ray film due to attenuation of x-rays, and hence create the needed distinction, or contrast, between the organ to be diagnosed, and the surrounding tissues. Intravascular administration of ICM is currently widely applied for both diagnostic and therapeutic purposes. Unfortunately, administration of ICM is associated with adverse side effects in vulnerable patients (Morcos and Thomsen, 2001;Namasivayam et al., 2006;Tumlin et al., 2006). Contrast-induced nephropathy (CIN) remains one of the most important complications with the use of iodinated x-ray contrast media, and that forms the basis for this present thesis.

1.1 History of iodinated x-ray contrast media

The first x-ray contrast media were introduced in the early 1920's. Agents at that time were strontium bromide, thorium dioxide, sodium bromide and organic di-iodinated preparations (Kaperonis et al., 2005). Already more than fifty years ago an association between the use of a di-ionidated contrast medium (a di-iodinated pyridine derivative, iodopyracet) and renal impairment was reported (Davidson et al., 2006). In the 1950's di-iodinated preparations were replaced by tri-iodinated contrast media, namely the ionic monomers. Tri-iodinated contrast media were better tolerated, but severe side effects were still frequently observed. Almen (1969) proposed that much of the observed toxicity was due to their high osmolality, and he aimed at developing better tolerated ICM. He proposed experiments to improve the tolerance of ICM by reducing the osmotic pressure and by eliminating the electrical charge in the molecule. His ideas laid the foundation for the new generation of ICM. Non-ionic monomers were introduced in the 1980's. Many of the products developed at that time are still frequently

used in radiographic examinations (e.g. iopamidol, iohexol and iopromide). In addition to having a lower osmolality, these agents were associated with far fewer side effect in patients compared to ionic ICM (Gries, 1999). The third generation of ICM, non-ionic dimers, were introduced in the 1990's. Non-ionic dimers have the same osmolality as blood and are better tolerated than non-ionic monomers (Aspelin et al., 2003).

Of the radio-opaque atoms examined, only three have proven useful for x-ray contrast media; barium, bromine and iodine (Gries, 1999). Of these three, iodine is preferred. This is because iodine is highly effective in absorbing x-rays within the diagnostic spectrum and because of its chemical versatility. Iodine allows stable binding of a variety of atoms to organic molecules, and if iodine atoms are released from the contrast molecule they have a low toxicity compared to other metals (Speck, 1999).

1.2 Nomenclature of iodinated x-ray contrast media

All commercial ICM are derivatives of a tri-iodinated benzoic acid, *Figure 1*. ICM are historically divided in two classes; ionic and non-ionic ICM. ICM in both of these classes can be further divided into monomers and dimers. Monomers contain one tri-iodinated benzene ring, while dimers consist of two tri-iodinated benzene rings linked together by an aliphatic bridge.

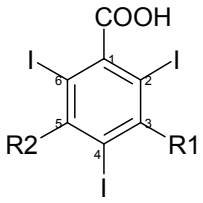


Figure 1 Tri-iodinated benzoic acid

Ionic monomers (*Figure 2*) consist of a cation (usually sodium, meglumine or a combination of both) and an anion (benzoic acid derivative) (Stacul, 2001). Ionic x-ray contrast media contain a carboxyl group at the 1st position of the benzene ring, and this carboxyl group dissociates into ions when dissolved in water or when it enters blood. The osmolality of such ICM are 4 to 7 times that of plasma (Stacul, 2001), and they are usually referred to as high-osmolal contrast media (HOCM).

By linking to ionic monomers with an aliphatic bridge, ionic dimers were developed in the

1980's (*Figure 2*). Their osmolality is reduced compared to ionic monomers, but they are still hyperosmolal compared to plasma.

Non-ionic monomers (*Figure 2*) have substituted the carboxyl group in the 1st position of the benzene ring with an alkanol side chain (Gries, 1999). These structures contain fewer osmotic active groups and hence, their osmolality is greatly reduced compared to the ionic ICM.

Both non-ionic monomers and ionic dimers are referred to as low-osmolal contrast media (LOCM). However, their osmolality is low compared to HOCM, but still approximately twice that of plasma (Stacul, 2001). The use of HOCM has been greatly reduced after the development of non-ionic monomers in the 1980's.

Non-ionic dimers (*Figure 2*) are two non-ionic tri-iodinated benzene rings linked together with an aliphatic bridge. ICM dimers of this type have the same osmolality as plasma (approximately 290 mOsm/kg) (Stacul, 2001), and are referred to as iso-osmolal contrast media (IOCM). So far only two non-ionic dimers have been developed: iotrolan and iodixanol.

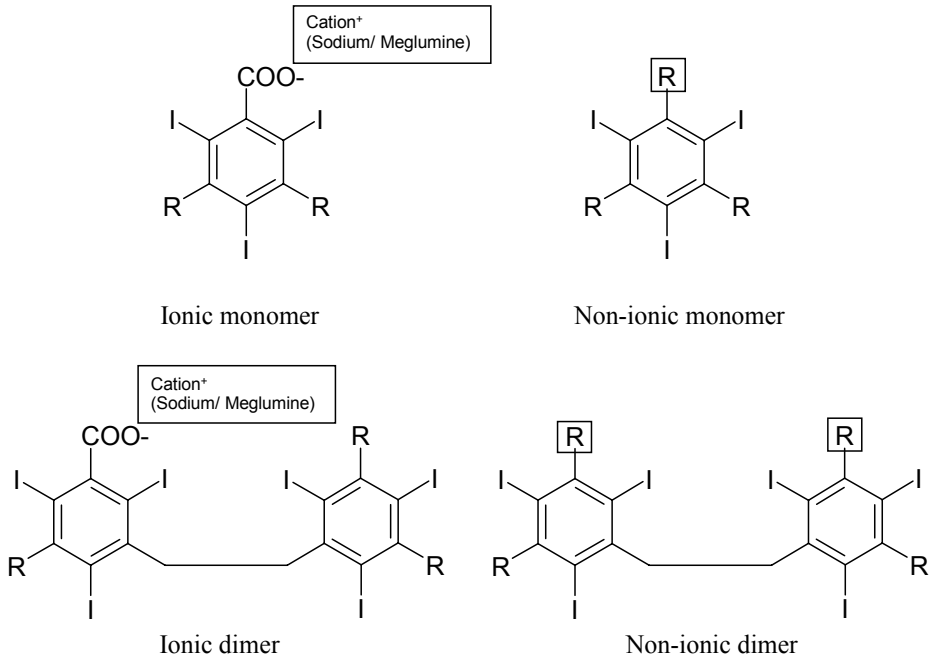


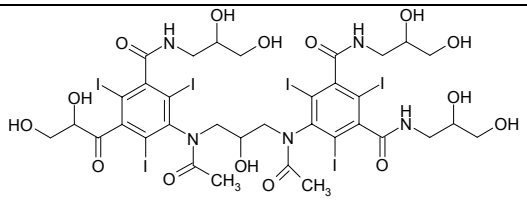
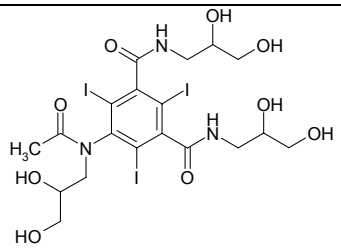
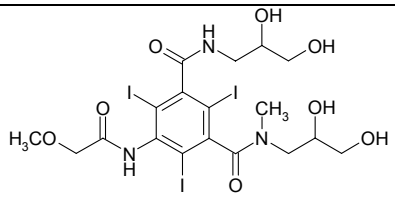
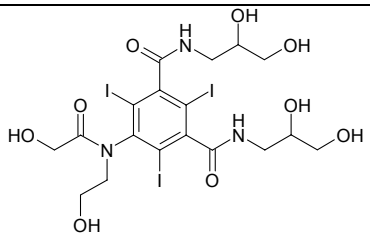
Figure 2 Classes of ICM

Taken together the evolution of ICM has focused on minimizing adverse side effects by eliminating ionicity, increasing the hydrophilicity, lowering the osmolality, and increasing the number of iodine atoms per molecule (Katzberg and Haller, 2006).

Osmolality depends on the number of molecules present in the solution. The osmolality of ICM has been reduced with non-ionic ICM which do not dissociate in solution and by dimer ICM containing 2 benzoic acid rings (Davidson et al., 2006).

In this master's thesis four ICM were selected for the experiments. Structure formulae and physicochemical properties of the four are presented in *Table 1*.

Table 1 Structure formulae and physicochemical properties for iodixanol, iohexol, iopromide, and ioversol.

X-ray contrast agent	Structure formulae	mOsm/kg undiluted	mOsm/kg (150 mg I/ml)	Molecular weight
Iodixanol		290 (320 mg I/ml)	350	1550
Iohexol		780 (350 mg I/ml)	559	821
Iopromide		610-620 (300 mg I/ml)	530	791
Ioversol		630 (300 mg I/ml)	531	807

Osmolality undiluted was adapted from Davidson et al.(2006), while osmolality at 150 mg I/ml (diluted in incubation medium) was measured on a Wescor 5520 XR Vapour Pressure Osmometer (Utah, USA), the solutions were measured in four replicates and the mean is reported. Molecular weights are adapted from Morcos (1999). Not that iodixanol is considered to be composed of two iohexol molecules.

1.3 Pharmacokinetics of iodinated x-ray contrast media

The pharmacokinetics of all the currently used ICM is similar. All of these agents have low lipid solubility, extremely low chemical reactivity with body fluids, and a relatively small molecular weight. Plasma half life of ICM in patients with normal renal function is approximately 1 to 2 hours. They are all in the class of compounds termed “extracellular tracers” with a bi-exponential decay curve. All ICM, with the exception of IOCM, are osmotic diuretics (Katzberg and Haller, 2006). Under normal physiological situations, close

to 100 percent of ICM are excreted through the kidneys, and less than 1 percent are excreted through extrarenal routes (liver, bile, small and large intestine, sweat, tears, and saliva) (Katzberg, 1997). The concentration of ICM within the kidney tubule depends on the concentration of the filtrate entering the kidneys and the degree of water reabsorption in the tubule. The ICM concentration in urine may be 50 to 100 times that in the plasma (Katzberg, 1997). This concentration of ICM in the kidneys make them especially vulnerable to the toxic effects of ICM.

1.4 Adverse reactions to iodinated x-ray contrast media

Ever since their introduction in the 1920's, ICM have been associated with adverse reactions in vulnerable patients. There has been a decrease in the number of incidences and severity as the second- (LOCM) and third generations (IOCM) of ICM have been developed, but severe reactions still occur.

Adverse reactions to ICM include general (acute and delayed) and organ specific adverse effects (contrast-induced nephrotoxicity, and cardiovascular, pulmonary, and neurotoxic effects) (Namasivayam et al., 2006). Acute reactions are normally defined as reactions that occur within one hour after administration of ICM, while delayed reactions occur more than one hour after administration of ICM (Christiansen et al., 2000;Morcos and Thomsen, 2001;Namasivayam et al., 2006;Webb et al., 2003).

The main focus in this thesis will be on the nephrotoxic effects of ICM.

1.4.1 Contrast-induced nephropathy

To achieve adequate contrast with ICM, large volumes (usually 50 – 200 ml) of highly concentrated ICM (300 – 350 mg I/ml) are injected intravenously or intra-arterially over a short time period. ICM are used in a much higher concentrations and doses than any other intravascular pharmaceutical (Christiansen, 2005;Katzberg and Haller, 2006). Due to the high administration dose, the kidneys as the almost exclusive excretion route, and concentration of ICM in the kidneys, it is not surprising that adverse renal effects occur.

Contrast-induced nephropathy (CIN) remains one of the most important complications with the use of ICM (Rihal et al., 2002). CIN has been reported to be the third-leading cause of acute renal failure (ARF) in hospitalized patients (Waybill and Waybill, 2001), accounting for 10 % of total cases (Persson, 2005). Fortunately, most incidences of CIN are self limiting and

resolve within 1 – 2 weeks. Permanent renal damage is rare and occurs only in a very few instances (Morcos and Thomsen, 2001).

Definition

The definition of CIN includes three necessary components; (1) an absolute or relative increase in serum creatinine values relative to the baseline value; (2) a temporal relationship between the rise in serum creatinine and administration of ICM; (3) the elimination of an alternative explanation for the renal impairment (Mehran and Nikolsky, 2006).

Several definitions exist, but most commonly CIN is defined as an acute impairment of renal function characterized by an absolute increase in serum creatinine concentration of at least 0.5 mg/dl (44 μmol/l), or by a relative increase of at least 25 % from baseline value occurring within 3 days after ICM administration and in the absence of an alternative aetiology (Aspelin et al., 2003;Mehran and Nikolsky, 2006;Morcos and Thomsen, 2001).

Clinical manifestation

An increase in serum creatinine and a decrease in creatinine clearance reflects the decrease in glomerular filtration rate (GFR) observed in patients with CIN (Thomsen and Morcos, 2003). The serum creatinine value usually increases within 24 to 48 hours after administration of ICM, reaching a peak value at 3 to 5 days, and usually returns to baseline values within 7 to 10 days (Waybill and Waybill, 2001). In some patients the return to baseline serum creatinine levels might take up to 3 weeks (Mehran and Nikolsky, 2006).

Mild proteinuria and oligouria may also be observed in CIN patients. Most patients are non-oligouric, with the exception of patients with pre-existing advanced chronic renal failure. However, extensive proteinuria is a common feature of CIN (Thomsen and Morcos, 2003). Urinary enzyme activity is also fond to increase after administration of ICM (Morcos and Thomsen, 2001).

Risk factors of contrast-induced nephropathy

Risk factors for CIN include both patient related factors and ICM related factors (Morcos, 1999). Pre-existing renal impairment and chronic kidney disease (estimated glomerular filtration rate (eGFR) < 60 ml/min/1.73 m^2) are universally considered to be the most important risk factors for CIN (McCullough et al., 2006;Mehran and Nikolsky, 2006;Waybill and Waybill, 2001). Furthermore, diabetes (type 1 and type 2) is also considered to be an important risk factor for CIN. Renal impairment is often found in association with diabetes,

and it is known that patients with diabetes and renal impairment are a group with a high risk of CIN (McCullough et al., 2006;Mehran and Nikolsky, 2006;Waybill and Waybill, 2001). Other patient related risk factors include volume depletion, pre-procedural haemodynamic instability, cardiovascular disease, age (> 70 years), and co-administration of nephrotoxic drugs (e.g. NSAIDS, cyclosporine and aminoglycosides) (Barrett and Parfrey, 2006;Lameire, 2006;McCullough et al., 2006;Mehran and Nikolsky, 2006;Morcos and Thomsen, 2001;Waybill and Waybill, 2001).

ICM related risk factors are the administered dose (high or low dose, simple or multiple administrations), the type of ICM (HOCM, LOCM or IOCM) and the route of administration (procedure specific).
Today there is no longer a question about using LOCM in preference to HOCM in patients at risk. LOCM are associated with lower toxicity compared to HOCM and hence, it is recommended to use LOCM (Davidson et al., 2006;Morcos and Thomsen, 2001).
Intuitively IOCM should pose a lower risk of CIN in patients at risk. Some authors claim that there is conflicting evidence whether IOCM are better tolerated than LOCM, and that more research is required (Morcos and Thomsen, 2001). While others conclude that IOCM are better tolerated than LOCM, and that IOCM should be used in patients with chronic kidney disease, particularly in those with diabetes (Davidson et al., 2006).

The volume of the ICM administered reflects a compromise between the safety of the patient and the need to optimize the visualization. The risk of CIN is dose-dependent within each contrast class (Davidson et al., 2006). There exists good evidence that the administered dose is a risk factor and formulae exist which can be used to adjust the dose of ICM to reduce the risk of CIN (Cigarroa et al., 1989). There is supporting evidence for this, as when the formula is applied, and the calculated dose is not exceeded, fewer patients at risk develop CIN (Waybill and Waybill, 2001). An administered dose over 100 ml in patients at risk has been associated with a higher rate of CIN (Davidson et al., 2006). Even a low dose of ICM (approximately 30 ml) may be sufficient to induce CIN in high risk patients and hence, there is no threshold volume below which CIN does not occur (Davidson et al., 2006).

It has been reported that pre-existing risk factors are additive and that patients with the presence of multiple pre-existing risk factors can be at very high risk of developing CIN (McCullough et al., 2006;Mehran and Nikolsky, 2006).

Incidence

The reported incidence of CIN is highly variable. This is mainly due to a lack of consensus in the definition of CIN, the presence and difference in pre-existing risk factors of the patient groups in the study, the type and dose of ICM administered, imaging procedure, and the length of follow-up (Lameire, 2006).

Development of CIN is rare in patients without pre-existing renal failure (Lameire, 2006;Mehran and Nikolsky, 2006;Morcos and Thomsen, 2001;Namasivayam et al., 2006;Waybill and Waybill, 2001). In patients with pre-existing renal impairment, CIN is more frequently reported, and the poorer the renal function the higher the incidence of CIN (Morcos, 1998). The incidence of CIN increases further as the number of risk factors in the patient increases. Incidences of CIN in patients at high risk varies and are reported up to 50 % (Morcos, 1998;Waybill and Waybill, 2001).

Pathophysiology of contrast-induced nephropathy

Currently the pathophysiology of CIN is poorly understood and little is known about the exact underlying mechanism. Several mechanisms have been proposed, and haemodynamic effects, direct tubular toxicity, and oxidative stress are considered the most important pathways for the pathogenesis of CIN (illustrated in *Figure 3)*. It is widely accepted that a combination of these various mechanisms leads to CIN (Persson and Tepel, 2006;Tumlin et al., 2006). Unfortunately, it is difficult to distinguish the true ICM toxicity from the secondary effects of renal ischemia, which may cause cellular injury associated with increased production and decreased removal of oxygen free radicals (Waybill and Waybill, 2001).

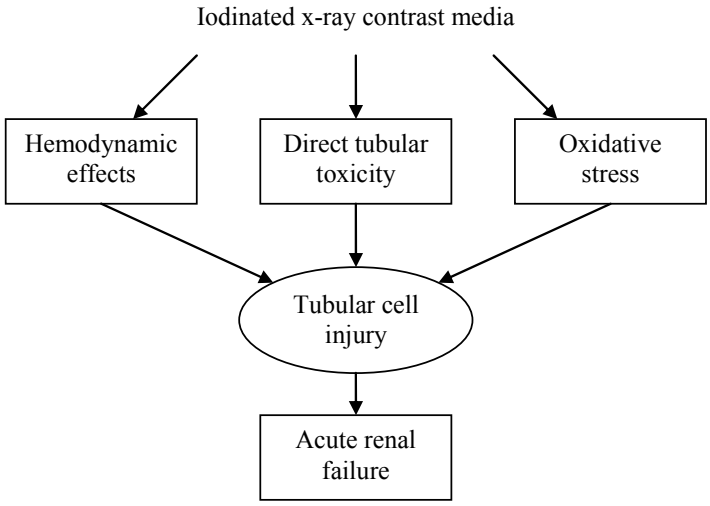


Figure 3 Schematic illustration of proposed pathways leading to CIN

Haemodynamic alterations

All ICM are vasoactive substances which have vasodilator effects in most vascular beds (Kaperonis et al., 2005). After administration of ICM, a biphasic haemodynamic response occurs; intra-arterial administration of ICM induces an initial vasodilation followed by a prolonged vasoconstriction (Tumlin et al., 2006). This biphasic reaction observed is far too transient and minimal to induce ischemic damage (Katzberg, 2005). The exact underlying mechanism for the vasoconstriction is not known, but the role of vasoconstrictors and vasodilators has been widely examined.

Of the evaluated vasoconstrictors, endothelin and adenosine have received most attention. Both vasoconstrictors are shown to be elevated after ICM administration and might be important in the pathogenesis of CIN by inducing a prolonged vasoconstriction which leads to additional injury (Kaperonis et al., 2005;Tumlin et al., 2006).

Nitrogen oxide (NO) is a potent vasodilator produced from L-arginine by NO synthase. Under normal conditions NO is released continuously and keeps the renal vasculature in a dilated state (Morcos, 1999). *In vitro* studies have shown that ICM causes a reduction in NO production, but this has not been confirmed in human studies (Tumlin et al., 2006). It has been stated that patients who are vulnerable to CIN already have an impairment in their endogenous vasodilator production and hence, the pre-existing status of the patient, not the ICM molecule, is responsible for the observed vasoconstriction (Morcos, 1999).

Direct tubular injury

Increased urinary excretion of lysosomal enzymes and small molecular weight proteins, both of which are non-specific markers of tubular damage, have been observed in patients after the administration of ICM (Waybill and Waybill, 2001).

Direct injury is also confirmed in different *in vitro* models; cellular energy failure, a disruption of calcium homeostasis, a disturbance of tubular cell polarity, apoptosis, altered cellular metabolism, pathological changes, and intracellular enzyme release are among the observations reported (Haller and Hizoh, 2004;Waybill and Waybill, 2001). It is difficult to distinguish the true ICM toxicity from the secondary effects. The molecular mechanisms of direct cytotoxicity are still unclear, but oxidative stress has been implicated (Haller and Hizoh, 2004).

Oxidative stress

The role of reactive oxygen species (ROS) (e.g. superoxide anion, hydrogen peroxide, hydroxyl radical, peroxynitrite and singlet oxygen) are often discussed in the pathogenesis of CIN (Katzberg, 2005;Tumlin et al., 2006). It is hypothesized that leucocytes, macrophages, and renal mesangial cells produce and release ROS, which in turn can lead to tubular injury (Kaperonis et al., 2005). Organ injury may occur when ROS exceed the antioxidant reservoir of the patient. The antioxidant reservoir decreases with increasing age and this makes elderly patients more susceptible. Increased renal oxidative stress is also present in patients with chronic renal failure and in diabetic patients, and that makes these patients more susceptible for the nephrotoxic effect of ICM (Tumlin et al., 2006).

Other possible mechanisms

Some studies report high incidence of renal retention of ICM in patients with impaired renal function, elderly patients, or both. A correlation between the degree of renal retention observed 24 hours after ICM administration and the onset of CIN has been reported (Tumlin et al., 2006). Renal retention of ICM may be related to CIN and may facilitate early detection of patients at high risk of CIN development.

It is accepted that the osmolality of the ICM and hence the osmotic load delivered to the kidneys may play a critical role in the pathogenesis of CIN (Mehran and Nikolsky,

2006;Tumlin et al., 2006), but it is not known whether this is a direct or indirect effect (Tumlin et al., 2006).

Biomarkers of contrast-induced nephropathy

Currently there is a lack of suitable biomarkers of CIN both in clinical and experimental settings. Most commonly, estimates of GFR (e.g. creatinine clearance) are used as a marker for CIN. Enzymuria and proteinuria following administration of ICM are reported, but no connection between these markers and the reduction in GFR has been established (Thomsen and Morcos, 2003). Still, enzymuria and proteinuria may be important as research tools in animal models and possibly also in clinical settings.

Among the most frequently used biomarkers to evaluate renal function is GFR. Ideally GFR is calculated as renal clearance of a substance which is exclusively eliminated by the kidneys, freely filtered by the glomerulus and neither reabsorbed nor excreted in the kidneys, i.e. inulin (Sands and Verlander, 2005) or ICM (Almen, 1994;Sterner et al., 1996). To evaluate GFR in this way is complex, time consuming, and might be further deleterious for patients with pre-existing kidney failure. Creatinine has several properties which make it a good estimator for GFR. It is an endogenous molecule which has a relatively stable concentration in plasma, is freely filtered by the glomerulus, and is excreted almost exclusively through the kidneys. However, creatinine undergoes tubular secretion by both the organic anion and cation pathways in the proximal tubule in most species, making it imperfect as an estimator for GFR (Hart and Kinter, 2005). However, compared to more accurate and complex methods (e.g. inulin clearance) creatinine clearance can be considered an acceptable compromise between accuracy and complexity for evaluation of GFR (Idee and Bonnemain, 1996).

Creatinine clearance is widely used to estimate GFR and can be calculated from serum and urine creatinine concentrations. An estimation of GFR (eGFR) can easily be calculated from serum creatinine concentration with the Cockcroft-Gault formula or the Modification in Diet in Renal Disease equation (Mehran and Nikolsky, 2006).

Serum creatinine is the most frequently used parameter to monitor renal function in patients at risk before administration of ICM (Thomsen and Morcos, 2003), and it is also commonly used as a marker of CIN after administration of ICM in clinical experiments (Aspelin et al.,

2003;Rudnick et al., 1995). Measurements of serum creatinine is a less sensitive parameter than GFR, since serum creatinine will not be affected unless GFR has been decreased by over 50 % (Idee and Bonnemain, 1996;Krause, 1998). For patients with pre-existing renal impairment it is not sufficient to measure serum creatinine as creatinine varies with age, muscle mass, diet and gender (Mehran and Nikolsky, 2006).

Enzymuria and proteinuria are among the clinical features of CIN. Several studies have reported on the effects of contrast media on various enzymes and proteins which are markers of glomerular and tubular functions. These markers include N-acetyl-β-D-glucosaminidase (NAG), γ-glutamyl transferase (GGT), alkaline phosphates, 3-nitrotyrosine, β_2-microglobulin, adenosine deaminase binding protein, and proteinuria (Tumlin et al., 2006).
No relationship between enzymuria and the reduction in GFR after administration of ICM has been established, and the detection of urinary enzymes following ICM administration is presumed to be of little importance to the clinical assessment and management of CIN (Thomsen and Morcos, 2003). Even so, both enzyme and protein markers are considered important research tools and may possibly lead to future diagnostic and therapeutic targets in CIN prediction and management (Tumlin et al., 2006).

1.5 *In vitro* experiments

The direct cytotoxicity of ICM is best studied in various cultured renal cells in the absence of confounding variables because *in vivo* experiments can not exclude interaction with hypoxia and other systemic mechanisms. In general, the toxic effects on renal cells observed *in vitro* comprise cellular energy failure, a disruption of calcium homeostasis, a disturbance of tubular cell polarity, and apoptosis (Haller and Hizoh, 2004).

In vitro experiments can be performed either on cultured primary cells or cell lines. Evaluation of nephrotoxic effects of ICM are mostly performed on either glomerular cell types or renal tubular epithelial cell types. The most frequently used cell line is Madin Darby Canine Kidney (MDCK), a cell line derived from the dog, which display features of a distal tubular cell phenotype. Also frequently used, but somewhat less than MDCK, are LLC-PK1 cells (porcine kidney cell line) which have a proximal tubular cell phenotype (Haller and Hizoh, 2004). In addition, HK-2 cells (human kidney proximal tubule cell line) and HRPTE

cells (primary cells from human proximal tubule) have also been used (Hardiek et al., 2001;Jenq et al., 1999;Zager et al., 2003).

Several *in vitro* experiments have been conducted to compare different types of ICM (comparing ICM within one class or between classes) and to evaluate effects of physicochemical properties. *In vitro* studies of this type confirm what has been observed in patients and in animal models, i.e. the toxicity is related to the osmolality of the ICM as LOCM have considerably less toxic effects than HOCM on various cell types, and the observed toxicity is dose dependent (Haller and Hizoh, 2004). The ionic force of the molecule seems to be irrelevant to the toxicity (Haller and Hizoh, 2004). Studies which imply that the direct toxicity is not only dependent on the osmolality of the ICM have been reported. The reason for this hypothesis to be proposed was the observation that mannitol, when tested at equal osmolality to the contrast agents, induced less toxicity (Haller et al., 1997;Hizoh et al., 1998).

Recently, studies which go into much more detail in regards to the possible underlying mechanisms of CIN are reported (Itoh et al., 2006;Yano et al., 2003;Yano et al., 2004;Yano et al., 2005). Yano et al. (2003) were the first to report on the cellular signalling cascade that could lead to the observed toxicity. The study showed that ICM-induced cell death involves caspase 3 and caspase 9, and to a lesser extent caspase 8, and that bax (an apoptosis promoter) mRNA was enhanced while bcl-2 (an anti-apoptotic protein) mRNA was reduced. These findings indicate that apoptosis might be important for the *in vitro* toxicity induced by ICM.

1.6 Animal models of contrast-induced neprohropathy

1.6.1 Renal insult

Currently there is a lack of a suitable animal model for CIN. The pathophysiology of CIN is not fully understood and is likely to be multifactorial, and because of this, it is difficult to create an animal model which resembles human CIN.

An *in vivo* model should fulfil one major condition; the course of the experimental nephropathy should be comparable to the nephropathy caused by ICM in humans (Idee and

Bonnemain, 1996). The ideal situation would be to have a model in which several factors, when given alone, do not cause injury, but when combined give a reproducible renal injury. A model of that type is more clinically relevant than any single insult model. Predisposing factors should be the same as in humans and a sustained reduction in GFR should be accomplished. Prophylactic approaches confirmed in humans should attenuate CIN in the model, and histopathological lesions in the animals should be the same as those observed in human CIN (Idee and Bonnemain, 1996).

CIN is difficult to induce in healthy animals (Morcos, 1999), with the exception of rabbits that seem to be more susceptible. Other animals, especially rats, seem to tolerate extremely high doses of ICM without any effect on renal function (Krause, 1998). Despite the high tolerance, healthy animals have been used in CIN studies. These animals have been injected with extremely high doses of CIN to induce some renal impairment. However, whether the renal toxicity induced in these animals after injection of a large volume of ICM is identical to human CIN is not known.

Animal models with renal impairment have been developed to resemble the patients at risk. Three major approaches have been used to induce renal impairment in animal models of CIN; inducing ischemic damage, dehydration of the animals, and inducing renal impairment by administration of nephrotoxic agents.

In principle, all agents that are nephrotoxic can be used to induce renal impairment. Agents used in CIN models include antibiotics (e.g. gentamicin (Idee et al., 1995;Thomsen et al., 1990b;Thomsen et al., 1991;Thomsen et al., 1993) and adriamycin (Uchimoto et al., 1994), cisplatin (Thomsen et al., 1995), glycerol (Thomsen et al., 1989a) and cyclosporine A (Thomsen et al., 1989b).

It is also possible to use diabetic rats. These can be purchased from suppliers, or diabetes can be induced in normal rats by administration of streptozotocin (toxic to the insulin-producing beta cells of the pancreas in mammals) (Yen et al., 2006).

The sensitivity of rats to ICM seems to be rather low even after multiple renal insults (Idee and Bonnemain, 1996). Hence, high doses are still necessary to induce CIN.

Animal models can be divided in two “categories” based on their design and aims; some models aim at improving the current understanding of CIN, while others aim at comparing ICM of different classes to evaluate their physicochemical properties (Idee and Bonnemain, 1996). Caution should be taken when results from the latter category are extrapolated to try to answer questions from the first category.

1.6.2 Animal species

The animal species most widely used in this field are rats. Rats are easy to handle, can be housed alone in metabolic cages and thus facilitate urine collection, standardized rat strains allow comparison between laboratories, and several strains with metabolic and/or physiologic abnormalities are available (Idee and Bonnemain, 1996). In addition to these advantages, rats are frequently used because their renal physiology is well known (Krause, 1998).

Rabbits are more sensitive than rats to ICM induced enzymuria and increase in serum creatinine and blood urea nitrogen, at least in healthy animals (Idee and Bonnemain, 1996). In a comprehensive study Petterson et al., (2002) have compared several ICM in a rabbit model.

Dogs have been used in studies evaluating the effect on GFR and in investigations aimed at the selection of prophylactic drugs because dog GFR more resembles human GFR than the rat (Krause, 1998). A clinically relevant model for studying the renal effects ICM is dogs with congestive heart failure induced by eight days of pacing the dogs at a ventricular rate of 250 beats per minute (Kaperonis et al., 2005).

The species which has kidneys that resemble the human kidney the most are the pig. However, relatively few studies on renal effects after ICM administration have been preformed on this species (Elmstahl et al., 2002;Elmstahl et al., 2004;Krause, 1998)

1.7 Summary

Nephrotoxic side effects remain a problem associated with administration of ICM. However, little is known about the underlying mechanism of the renal toxicity. The only way to improve our current understanding of problems related to administration of ICM is through *in vitro*, animal experiments and clinical experiments. However it is important to keep in mind

that *in vitro*, *ex vivo* and *in vivo* models should not be compared as they do not provide the same information. They are intended to improve our understanding of different aspects of the same problem (Idee and Bonnemain, 1996), and together they can improve our understanding.

1.8 Aims of the study

This master's thesis was performed to investigate the nephrotoxic effects of ICM *in vitro* and *in vivo*, with three main aims:

1. To evaluate the toxicity of four ICM with different physicochemical properties in cultured cells.

The test system was a rat kidney cell line with epithelial-like morphology (NRK 52-E), and which has not, to my knowledge, been used for testing ICM toxicity. The ICM selected were iodixanol (IOCM), iohexol (LOCM), iopromide (LOCM) and ioversol (LOCM). All ICM selected are commercial available ICM which are frequently used for radiographic examinations.

2. To develop an animal model of impaired renal function.

The second aim of the study was to develop a model which could be used to evaluate *in vivo* nephrotoxic effects of ICM, and possibly be used to screen novel ICM candidates in the future. The rat was chosen as a suitable species, and gentamicin (an antibiotic known to cause renal toxicity) selected as the agents to induce renal impairment. The rat-gentamicin model developed in this master's thesis aimed to improve previously reported models (Idee et al., 1995;Thomsen et al., 1990b;Thomsen et al., 1991;Thomsen et al., 1993;Uchimoto et al., 1994).

3. To evaluate potential biomarkers of contrast-induced renal injury

Currently there are no generally accepted reliable biomarkers for CIN which can be used in both animal models and patients. Recently several novel biomarkers have been proposed to detect early renal damage, but only a minority of them have been tested in studies on nephrotoxicity caused by ICM. The developed rat-gentamicin model was used as the test system to evaluate potential biomarkers of renal damage caused by ICM. Some novel and some traditional serum and urinary biomarkers of renal injury were selected. Creatinine and

cystatin C were selected as serum biomarkers, and creatinine, N-acetyl-β-D-glucosaminidase (NAG, a lysosomal enzyme), γ-glutamyl transferase (GGT, a brush border enzyme), alpha glutathione-S-transferase (α-GST, a cytosolic protein), cystatin C and total protein were selected as urinary biomarkers. In addition, urinary epithelial cell counting was chosen as a biomarker of renal injury.

2. Background

ICM are associated with CIN in vulnerable patients. The current understandings of the underlying mechanisms for CIN, the population of patients at risk, and prophylactic interventions, are increasing, but still many questions remain unanswered. The aim of this master's thesis was to evaluate the effects of iodinated x-ray contrast media *in vitro* and *in vivo*, with emphasis on the nephrotoxic side effects of ICM.

2.1 *In vitro* experiments

To evaluate the toxicity of four ICM with different physiochemical properties *in vitro*, three cell death or cell viability assays were selected; the trypan blue exclusion assay, the MTT assay, and cell death assessment on formalin fixed cells. An assessment of cell morphology was included to supplement the selected tests, and an analytical method, inductively coupled plasma atomic emission spectrometry (ICP-AES), was used to measure iodine in cell extracts.

2.1.1 Cell death and cell viability assays

An efficient and cost effective method to study cell death is by using trypan blue. The trypan blue exclusion assay is one of the most commonly used methods to assess cell death after ICM exposure and experiments using trypan blue is frequently reported (Andersen et al., 1994;Haller et al., 1997;Hardiek et al., 2001;Heinrich et al., 2007;Schick and Haller, 1999;Schick et al., 2002).

The MTT assay is a well established cell viability assay. It is rapid, simple, accurate, versatile, and gives reproducible results (Mosmann, 1983). The MTT assay is commonly used to assess cell viability after ICM exposure (Duan et al., 2006;Hardiek et al., 2001;Heinrich et al., 2007;Heinrich et al., 2005;Zager et al., 2003).

Cell morphology is a useful approach as a supplement to other cell death and cell viability tests. Cell morphology gives a picture of what the cells actually look like, and hence can be used to confirm or disprove results from cell death or cell viability tests. In addition, cell morphology assessed after ICM exposure makes it possible to detect features which can not

be elucidated by any cell death or cell viability test (e.g. vacuolation, cells that are in progress of dying but not yet dead, the state of confluence, etc). Both sophisticated (Zhang et al., 2000) (time laps video microscopy) and basic (Hizoh et al., 1998) (Giemsa stain, a mixture of methylene blue and eosin) cell morphology methods have previously been described in the literature. In this present thesis, both quantitative (cell counting) and qualitative (description) morphology was assessed with hematoxylin stained cells.

2.1.2 Internalization of iodinated x-ray contrast media

Renal retention of ICM has been observed in patients (Tumlin et al., 2006) and in animal studies (Dobrota et al., 1995;Walday et al., 1995) after administration of ICM. Furthermore, uptake of ICM has also been shown *in vitro* in cultured cells (Andersen et al., 1994). High doses of ICM in experimental animals leads to rapid development of large vacuoles in the epithelial cells in the proximal tubule of the kidney. This process was first described as osmotic nephrosis, but since then it has been shown that the vacuolation in the epithelial cells is more pronounced with IOCM and LOCM than with HOCM (Dobrota et al., 1995). Hence, the vacuolation can not be attributed to osmotic effects of ICM. It has been suggested that the higher viscosity of dimers increases the backpressure in the nephron as IOCM allows higher tubular concentrations than HOCM. This highly viscous filtrate might have a longer transit time through the tubules and hence providing increase opportunity of ICM uptake (Dobrota et al., 1995).

Dobrota et al. (1995) reported the vacuoles as giant lysosomes containing the intracellular retained ICM (and proteins) taken up by fluid phase endocytosis (or pinocytocis) in rats. The observed vacuolation was not associated with any obvious impairment of the tubular function. Andersen et al. (1994) showed that vacuolation also appears in cultured cell lines (MDCK and LLC PK-1). After exposure to ICM, both cell lines showed an extensive vacuolar apparatus consisting of endocytic vacuoles and lysosomes. The vacuoles were shown to contain an electron dense granular precipitate when examined by electron microscope, and this was explained by ICM uptake and retention in vacuoles. Vacuolation has not been associated with loss of function, osmolality, viscosity or overall hydrophilicity of the ICM (Hardiek et al., 2001).

To my knowledge, internalization of ICM has not been previously tested *in vitro* with ICP-AES analyses as presented in this thesis. The study design included exposure of ICM to NRK 52-E cells, preparation of cell extracts at the termination of exposure, ICP-AES analyses to measure the iodine concentration in the cell extracts, and measurement of proteins in the same cell extracts to serve to normalize the measured iodine concentrations.

2.2 *In vivo* and *ex vivo* experiments

2.2.1 Animal model of renal impairment - the rat-gentamicin model

The rat-gentamicin model is previously described in the literature and it can be used for different types of nephrotoxic studies. The model utilises the nephrotoxic properties of gentamicin to induce the required degree of renal failure. High doses of gentamicin (40 mg/kg bw or more) are necessary in animals to rapidly induce extended cortical necrosis and overt renal dysfunction (Mingeot-Leclercq and Tulkens, 1999).

Aminoglycosides like gentamicin exert their bactericidal effect by binding to prokaryotic ribosomes and consequently blocking the ribosomal translation complex leading to mistranslation. This effectively inhibits or impairs protein synthesis and thus leads to bacterial death (Sundin and Molitoris, 2005). Aminoglycoside antibiotics are the most commonly used antibiotics for gram negative bacterial infections (Nagai and Takano, 2004). Aminoglycosides are low protein binding drugs that are not metabolized in the body and which are freely filtered through glomerulus (Nagai and Takano, 2004). With a half life of 30 to 90 minutes in rats, gentamicin is rapidly excreted (Sundin and Molitoris, 2005). The kidneys are susceptible since they are the exclusive excretion route for aminoglycosides (Sundin and Molitoris, 2005). Most of the administered dose is excreted in the urine, but a small amount (5 %) is taken up and accumulated in the S_1 and S_2 segments of proximal tubule. This uptake is thought to lead to the observed toxicity (Mingeot-Leclercq and Tulkens, 1999). The current model for uptake in renal cells is described by Moestrup at al. (1995). The model suggests that aminoglycosides binds to acidic phospholipids at tips of microvilli in the proximal tubule of the kidney. From there the acidic phospholipid-aminoglycoside complex diffuses within the plane of the membrane to a coated pit region at the base of the microvilli. Once the complex reaches the coated pit, the aminoglycoside is transferred to megalin, a large receptor

also known as gp330, by a mechanism not yet described. Following this, it is taken up into the cell via the receptor mediated endocytic pathway. Coated pits, coated and uncoated vesicles, endosomes and lysosomes are involved in the intracellular trafficking of aminoglycosides, and there is evidence that gentamicin transits through and accumulates in these structures (Sundin and Molitoris, 2005).

Histopathological studies strongly indicate that tubular necrosis is the primary cause of functional aminoglycoside toxicity (Mingeot-Leclercq and Tulkens, 1999). Many mechanisms are proposed, but the exact underlying mechanism for the nephrotoxicity of aminoglycosides remains unknown. Due to a sudden onset of renal impairment and gross necrosis that then occurs, it is difficult to differentiate between the primary and secondary causes of nephrotoxicity (Sundin and Molitoris, 2005).

When extended cortical necrosis and overt renal dysfunction is induced after administration of high doses of gentamicin in animals, a variety of structural, metabolic and functional changes are observed in tubular cells. Several of these changes have been claimed to be responsible for cell death and renal dysfunction. These changes comprise inhibition of protein synthesis, modulation of gene expression, mitochondrial alterations, inhibition of enzymes, impairment and inhibition of membrane transport systems and more (see Mingeot-Leclercq and Tulkens (1999) for a review). The role of ROS has also been pointed out as an underlying mechanism for the observed toxicity (Basnakian et al., 2005), but it is uncertain whether this is a primary or secondary mechanism of toxicity.

The rat-gentamicin model developed in this master's thesis aimed to improve previously reported models (Idee et al., 1995;Thomsen et al., 1990b;Thomsen et al., 1993;Uchimoto et al., 1994).

2.2.2 Biomarkers of renal injury

Assessment of renal function can be preformed by measuring serum or plasma parameters, urine parameters, or both, by urine cytology and by histological examination. Plasma biomarkers can provide information about the level of renal perfusion and functional nephron mass, while urine biomarkers give information on the kidney function (Hart and Kinter, 2005). In addition, urine biomarkers can indicate the initial site and the severity of kidney

damage (Price, 2002).

Plasma and serum markers serve mainly as estimators of GFR (e.g. creatinine clearance), but can also be used directly as a biomarker. Commonly used plasma and serum markers are creatinine and urea. Limitations to the utility these markers for renal function exists (Hart and Kinter, 2005). The markers are insensitive due to the kidneys large functional reserve. Approximately 50 % of the renal function must be lost before the concentration of these markers rise (Price, 2002). In addition, they are very sensitive to any factor that alter renal perfusion. Both prerenal factors (e.g. volume depletion, blood loss) and postrenal factors (e.g. extravasation of urine to the peritoneal cavity) may cause elevations in the measured markers that do not reflect primary kidney injury (Hart and Kinter, 2005). Another limitation is that serum parameters can not be used to determine the origin of the damage within the tubule (Hart and Kinter, 2005).

Urinary tests are better than serum test to assess renal function. Urine is the main product of the kidneys, and hence parameters found in the urine are the best indicator of their function. Upon renal damage many proteins and enzymes are up-regulated to compensate for the induced damage, while others leak into the urine as a direct consequence of tubular damage.

Most of the commonly used proteins and enzymes are of high enough molecular weight to allow the extrarenal contribution to be ignored (Hart and Kinter, 2005). Many proteins and enzymes in the kidneys have a unique distribution along the nephron, and that makes it possible to indicate the initial site of injury (Price, 2002). The severity of renal damage can also be assessed by urinary markers, made possible by a knowledge of their location within the cell. Three major sites of release have been identified; the lysosymes, the brush-border, and the cytoplasm. A rule of thumb is that proteins and enzymes of cytoplasm and lysosyme origin indicates a more severe renal damage than brush-border enzymes and proteins (Hart and Kinter, 2005).

Histopathology allows renal tubular injury (which may be reversible), to be distinguished from altered renal function which, if not progressive, might be improved over a long period of time (Loeb, 1998). In addition to this, histopathology also gives an accurate image of what the kidney actually looks like and the extent and severity of the damage.

Currently, macroproteinuria, creatinine clearance and histological examination are used as a gold-standard for renal damage in clinical medicine, occupational health and in drug evaluation (Price, 2002). Over the last years several new and promising biomarkers of renal damage have been described (see Hart and Kinter (2005) for more details on renal biomarkers and their nephron segment origin in the kidney). Only a minority of these new biomarkers have been tested to establish a potential correlation to CIN.

In this thesis some novel and some traditional serum and urinary biomarkers of renal injury were selected. Creatinine and cystatin C were selected as serum biomarkers. Creatinine, N-acetyl-β-D-glucosaminidase (NAG, a lysosomal enzyme), γ-glutamyl transferase (GGT, a brush border enzyme), alpha glutathione-S-transferase (α-GST, a cytosolic protein), total protein and cystatin C were selected as urine markers. The selected biomarkers will be described in the following sections.

Creatinine

Creatinine is a muscle-derived by-product of creatine metabolism. Creatinine is produced and delivered to the plasma at a fairly constant rate. As the concentration in plasma increase, the synthesis declines. Hence, the day-to-day plasma creatinine concentration does not vary widely (Hart and Kinter, 2005). Upon renal damage the amount of creatinine that is filtered and excreted through the kidneys decreases, and hence the serum concentration rises. A significant increase in serum creatinine concentration is not detectible unless the kidney function has been reduced by approximately 50 % (Price, 2002). Therefore, creatinine is not considered to be a biomarker which detects early renal damages. Creatinine can be used as a biomarker on its own or be used as an estimator for GFR (creatinine clearance). Creatinine clearance is generally considered to be more accurate than serum creatinine to evaluate renal function (Idee and Bonnemain, 1996).

Cystatin C

Cystatin C is a cysteine protease inhibitor in the cystatin superfamily. Its biological function is the control of proteolytic enzymes (Mares et al., 2003). Cystatin C is a 13 kDa protein which is produced at a constant rate by most nucleated cells (Uchida and Gotoh, 2002). The gene for cystatin C has been localized to chromosome 20 (Olafsson, 1995). Cystatin C is freely filtered by the glomerulus (Uchida and Gotoh, 2002), reabsorbed by tubular cells and catabolized so that it does not return to the blood flow (Laterza et al., 2002). As it is

catabolized almost completely, only 0.5 % appears in the urine (Bökenkamp et al., 2006). Cystatin C is not secreted by tubular cells, and serum concentrations are independent of age, sex, body mass, and hydration status (Trof et al., 2006)

The urinary concentration of cystatin C is low, normally 100 µg/l in healthy individuals (Uchida and Gotoh, 2002). It is reported that the urinary concentration increases with the severity of renal damage. Studies have shown that urinary concentration of cystatin C in patients with renal tubular disorders increased approximately 200-fold (Uchida and Gotoh, 2002).

Cystantin C has many of the preferred properties of an endogenous estimator for GFR and has frequently been compared with creatinine for estimation of GFR. It has been shown that serum cystatin C performs at least as well as serum creatinine as a renal marker, and several studies suggest that serum cystatin C is more sensitive to small changes in GFR than serum creatinine (Laterza et al., 2002). Still, many reports show no superiority of cystantin C over serum creatinine. Cystatin C, like creatinine, has limitations, and therefore a recent review concluded that more studies are needed to evaluate the performance of cystatin C as an estimator of GFR (Zahran et al., 2007).

N-acetyl-β-D-glucosaminidase

NAG is a lysosomal enzyme involved in the breakdown metabolism of glycoproteins (Price, 1992). It is a widely distributed lysosomal enzyme located mainly in the renal proximal tubule. Elevated urinary NAG activity has been associated with various kidney injuries (Numata et al., 1997). NAG is used widely as an organ specific indicator of renal damage (Ragan and Weller, 1999), and considered a sensitive marker of renal diseases, an early warning of rejection after transplantation and a sign of drug-induced nephrotoxicity (Numata et al., 1997).

γ-Glutamyl transferase

GGT is one of six enzymes that function in the gamma glutamyl cycle. GGT is a membrane bound enzyme which catalyzes the transfer of gamma glutamyl groups from gamma glutamyl peptides (e.g. GSH) to other peptides, amino acids and water (Hoffmann and Solter, 1999). GGT plays a major role in the regulation of GSH and functions in the GSH transferase/GGT pathway that cleaves the gamma glutamyl moiety from GSH conjugates. The latter is

important for detoxification of xenobiotics and carcinogens by making them more water soluble and hence allowing excretion (Hoffmann and Solter, 1999).

The kidneys, pancreas and liver are the organs where the highest concentration of GGT is found in all species. GGT is also found in the spleen, lung, intestine, seminal vesicles, mammary gland, and the ciliary body, but in smaller amounts. GGT is generally found on the external surface of cells (Hoffmann and Solter, 1999).

In the kidneys, γ-glutamyl transferase is located in the brush-border of epithelial cells in the proximal straight and convoluted tubules (Loeb, 1998). GGT activity increases in urine during early renal tubular injury. The renal location of GGT, on the surface of proximal tubular epithelial cells, results in rapid release into the urine during cellular injury or normal cellular turnover (Price, 1982). GGT is useful as a diagnostic test of acute injury, but less useful with chronic injury (Hoffmann and Solter, 1999).

α-Glutathione S-transferase

Glutathione transferases (GST) are a family of proteins that function both as important detoxification enzymes and as intracellular binding proteins. GST catalyse the reaction between glutathione (gamma-gluatmylcysteine or GSH) and a number of electrophilic compounds and carcinogens, allowing them to be detoxified and excreted. As a binding proteins, GST bind to a number of amphipathic compounds such as bilirubin, fatty acids, steroids and xenobiotics that are transported intracellularly. Due to the latter function GST have also been named ligandins (Hoffmann and Solter, 1999).

Investigations have established that tubular epithelial cells of the kidney have relatively high concentrations of GST (Bruning et al., 1999). Several families of GST exists, and the alpha form is exclusively associated with the proximal and distal convoluted tubule of the kidney (Oberley et al., 1995). Of the cytoplasmic protein content in proximal tubular epithelial cells, the alpha form of GST comprises 2 % of the total (Bruning et al., 1999).

Urinary α-GST concentration is low under normal conditions. Upon damage to the plasma membrane, α-GST is released into the urine and its presence can thus be used as a biomarker of renal proximal tubular damage (Bruning et al., 1999). Urinary GST is specific for renal

damage as GST released from other organs is not filtered through the glomeruli (Bruning et al., 1999). When evaluated as a biomarker for monitoring fluoride nephrotoxicity, α-GST proved to be useful as a marker for early detection and for long-term evaluation of proximal tubular injury (Usuda et al., 1998).

Total protein

A small amount of protein in the urine is normal for most species. The presence of protein in the urine is a result of filtration through the glomeruli and incomplete reabsorption in the proximal tubule cells, from secretion into the urine, and from natural turnover of tubular epithelial cells (Hart and Kinter, 2005). Changes in levels and types of proteins present in the urine can provide information on the integrity of the glomerular filtration membrane, the endocytic capacity of the proximal tubule, and alterations in proteins synthesized and excreted in response to physiological stimuli (Hart and Kinter, 2005). As a general rule, markedly elevated proteinuria is indicative of glomerular disease, whereas low level proteinuria indicates tubular damage or very early low grade glomerular injury (Hart and Kinter, 2005).

Urine cytology

Examination of the urine sediment (urine cytology) may detect structural abnormalities of the urinary system and provide diagnostic information when compared to physiochemical and/or functional renal test. Hence, urine cytology should be a part of the routine urinalysis (Ragan and Weller, 1999).

Urine cytology is most commonly performed on a stained or unstained smear of fresh urine examined with bright field and/or phase-contrast microscopy. It is also possible to make permanent cytocentrifuge or paraffin embedded preparations of the urine sediment. Cytocentrifuge and Papanicolaou staining of urinary sediment result in preparations superior to those of conventional methods. This technique allows permanent preparations to be made, in contrast to temporary wet mounts (Ragan and Weller, 1999).

The formed elements of urine fall into four general types (Ragan and Weller, 1999): (1) host cells, which can be haematopoietic, epithelial or neoplatic cells; (2) casts, which can be hyaline, granular, red blood cell, white blood cell, epithelial, waxy, fatty, mixed, or pseudo-casts; (3) crystals, composed of phosphate, urate, oxalate, bilirubin, tyrosine, cysteine, cholesterol, or drugs; and (4) nonhost cells, e.g. bacteria, yeast, ova, or parasites.

Casts in small numbers are normal components of urine, but increased number of casts or presence of granules (cell or cellular debris) are indicative of tubular injury. The presences of renal epithelial cells (more than from natural turnover) in the urine strongly indicate tubular injury. The origin of bleeding or inflammation can sometimes be determined by urine cytology (Hart and Kinter, 2005). It is the number of each element present that is considered under evaluation, and as described briefly most elements are an indicator for a specific type of renal injury or the site of the injury.

3. Materials and methods

3.1 Chemicals

Table 2 Chemicals and reagents used throughout this thesis. Chemicals used on preparations that were preformed at the Veterinarian Institute (cytospin preparations of urinary sediment) are not listed.

Chemical/Reagent	Supplier
Ascorbic acid	Merck, Gremany
Creatinine Reagent	Beckman Coulter, USA
Cystatin C (human) ELISA kit	Alexis Biochemicals, USA
Dulbecco's Modified Eagles medium (DMEM)	Sigma-Aldrich, Norway
Dulbecco's Phosphate Buffered Saline	Sigma-Aldrich, Norway
Eosin Y solution	Sigma-Aldrich, Norway
Fetal Bovine sera (FBS)	Sigma-Aldrich, Norway
γ-Glutamyl Transferase (GGT) Reagent	Beckman Coulter, USA
Gentamicin (Garamycin, 40 mg/ml)	Schering-Plough, USA
Hematoxylin (Harris HTX)	HistoLab Products AB, Sweden
Histo-Clear® II	National Diagnostics, USA
Hydrochloric acid, 5 M	Merck, Gremany
Iodine, SPS standard	Holger Technology, Norway
Iodixanol (Visipaque®, 320 mg I/ml)	GE Healthcare, Norway
Iohexol (Omnipaque®, 350 ml I/ml)	GE Healthcare, Norway
Iopromide (Ultravist®, 300 mg I/ml)	Bayer Schering Pharma, Germany
Ioversol (Optiray®, 300 mg I/ml)	Tyco Healthcare, USA
Isopropanol	Arcus, Norway
L-Glutamine, 2 mM	Sigma-Aldrich, Norway
Micro BCA™ Protein assay	Pierce, USA
MTT [3-(4,5-dimetylthiozol-2-yl)2,5-diphenyltetrazolium bromide]	Sigma-Aldrich, Norway
N-Acetyl-β-D-Glucosaminidase (NAG) Standard	Roche Applied Science, Germany
Neutral buffered formalin (10 %)	Richard-Allan Scientific, USA
Non Essential Amino Acids	Sigma-Aldrich, Norway
Penicillin/Streptomycin solution	Sigma-Aldrich, Norway
Pentothal-Natrium® (Thiopental)	Hospira, The Netherlands
Rat α-GST EIA	Biotrin, Ireland
Scandium, SPS Standards	Holger Technology, Norway
Total Protein Kit	Sigma-Aldrich, Norway
Triton-X 100, Sigma Ultra	Sigma-Aldrich, Norway
Trypan blue solution, 0.4 %	Sigma-Aldrich, Norway
Trypsin-EDTA solution, 1x	Sigma-Aldrich, Norway

3.2 Materials

A rat kidney epithelial cell line (NRK-52E) was purchased from European Collection of Cell Cultures. The cell line is cloned from a mixed culture of normal rat kidney cells, but has epithelial-like morphology.

Sprague Dawley rats (Bkl:SD) from B&K Sweden, were obtained through Scanbur BK AS (Norway).

3.3 *In vitro* methods

Cell death and cell viability after ICM exposure were assessed on the NRK 52-E cell line by use of three different methods: the trypan blue exclusion assay, the MTT assay and cell morphology.
Internalization of ICM in NRK 52-E cells was examined by ICP-AES analysis on cell extracts. Total protein content was measured in the same cells extracts samples by BCA micro protein assay (Pierce, USA), and used to normalize the measured iodine content to protein content (indirect cell measurement). In addition to the quantitative measurement of internalization of ICM by ICP-AES analysis, a qualitative description of the observed vacuolisation of ICM was performed when cell morphology was assessed.

3.3.1 Sterile working

All experiments preformed with the NRK 52-E cell line were conducted under sterile conditions in a laminar flow hood (KR-210 Safety Kojair, Finland). All equipment used was autoclaved (HM CLAVE™, Hirayama, Japan) or disinfected in 70 % ethanol (Shield Medicare, UK).

3.3.2 Cell culture

The NRK 52-E cells were cultured as recommended by the supplier, with minor modifications. The cells were routinely grown in 75 cm^2 cell culture flasks (Becton Dickinson, USA) in Dulbecco's Modified Eagles medium supplemented with 10 % fetal bovine sera, 1 % 2mM L-glutamine, 1 % non essential amino acids, and 1 % penicillin/streptomycin solution. Cells were maintained in a sterile incubator (CO_2 incubator

MCO-175M, Sanyo Electric co. Ltd., Japan) at 37 ºC with 5 % CO_2 under saturated humidity. The cell medium was changed every third or fourth day. Passages over 20 were discarded and replaced by cells from frozen stock stored in liquid nitrogen.

3.3.3 Trypan blue exclusion assay

Staining cultured cells with vital dyes, including trypan blue, is a common approach to quantify cell death. The principle of the trypan blue exclusion assay is that living cells exclude the dye and remain unstained, while dead cells are unable to exclude the dye, and hence stain intensely blue. Living and dead cells can therefore be distinguished by their colour when visualized by light microscopy.

Cell death was assessed after exposure to incubation medium (Dulbecco's Modified Eagles medium supplemented with 1 % 2mM L-glutamine, 1 % non essential amino acids and 1 % penicillin/streptomycin solution) (control), iodixanol, iohexol, iopromide, and ioversol for 1, 3, 6, 12 and 24 hours. All ICM were tested at a concentration of 150 mg I/ml (diluted in incubation medium).

Confluent NRK 52-E cells (normally 3 days after sub-cultivation) in a 6 well plate (NUNC, Denmark) were washed carefully with PBS and exposed in duplicate to incubation medium and ICM for the different exposure periods. At the termination of the experiment, the incubation medium and ICM were transferred to separate centrifuge tubes, to include possible detached dead cells. Trypsin-EDTA solution, 1 ml, was added to each well and the plate was placed in the incubator until the cells detached from the well. Following this, the Trypsin-EDTA solutions with cells were transferred to its respective centrifuge tubes along with 7 of ml fresh incubation medium. Fresh incubation medium was added to dilute the ICM sufficiently to avoid it being centrifuged down and preventing cell pellet formation in the bottom of the tube (ICM has higher density than cells in medium).

The tubes were centrifuged at 1000 rpm for 10 minutes (Spincron R Centrifuge, Beckman, USA). Supernatants were removed with careful suction by pipette and the pellets were resuspended in incubation medium (100 – 300 µl, depending on the size of the pellet). After proper and careful homogenization of the cell suspension, 50 µl was transferred to an Eppendorf tube and an equal volume of 0.4 % trypan blue solution was added. The trypan

blue cell suspension was incubated for one minute before approximately 20 µl suspension was transferred to a Bürker Türk chamber (Marienfeld, Germany). Light microscopic examination was preformed to distinguish and count living and dead cells.

Cell death was calculated and expressed as a percentage ((dead cells/total cells) × 100 %).

For each time point a minimum of 200 cells were counted in each duplicate for ICM and controls. Each experiment was repeated 5 times.

3.3.4 MTT assay

The MTT [3-(4,5-dimetylthiozol-2-yl)2,5-diphenyltetrazolium bromide] assay is based on the method originally described by Mosmann (1983), and conducted with modifications adapted from Sigma-Aldrich's MTT based *in vitro* toxicology kit. The assay measures mitochondrial dehydrogenase activity, and is therefore an indirect way of measuring cell death. MTT is a yellow tetrazolium salt that is actively transported into the cell and reduced to a purple formazane by-product by mitochondrial dehydrogenases (*Figure 4*). Only viable cells are capable of cleaving the tetrazolium ring in MTT. An increase or decrease in cell viability results in a corresponding change in the amount of formazane generated by mitochondrial dehydrogenases. The amount of formazane generated is directly proportional to the cell number over a wide range (Mosmann, 1983).

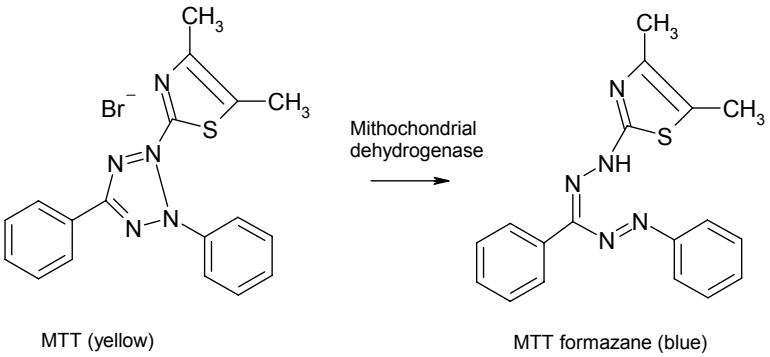


Figure 4 Reduction of MTT to its colored product

Cell viability was assessed after exposure to incubation medium (control), iodixanol, iohexol, iopromide and ioversol for 0.5, 1, 2, 3, 6, 12 and 24 hours. All ICM were tested at a concentration of 150 mg I/ml (diluted in incubation medium).

NRK 52-E cells were sub-cultured in 96 well plates (NUNC, Denmark) and grown to confluence (normally 3 days). When confluence was reached, the cell medium was removed from each well, and the incubation medium and ICM were added, 100 µl to each well. During the exposure time the microtitrer plates were kept in the incubator (37 ºC with 5 % CO_2 under saturated humidity).

At the end of the exposure time, 10 µl (10 % of the original volume in the well) of the MTT solution (0.05 mg/ml in PBS) was added to each well. The plate was placed in the incubator again for 4 hours incubation. At the end of the MTT incubation period, 100 µl MTT solubilization solution (10% Triton X-100 plus in 0.1 M HCl in anhydrous isopropanol) was added to each well. The plates were placed in an incubator at 37 ºC (Termaks, Norway) overnight for the formazan crystals to dissolve. The next day the absorbance was measured (VERSAmax™ Turnable Microplate reader, Molecular Device, USA) at 570 nm using 630 nm as reference.

Cell viability is expressed as a percentage of the control values; $OD_{sample} / OD_{control} \times 100\ \%$ = cell viability in % of control. Each experiment was repeated five to eight times.

3.3.5 Cell morphology

Cell morphology, both quantitative (counting dead cells) and descriptive (description of confluence, vacuolisation, etc.), was assessed in NRK 52-E cells after exposure to incubation medium (control), iodixanol, iohexol, iopromide and ioversol for 0.5, 1, 2, 3, 6, 12 and 24 hours. All ICM were tested at a concentration of 150 mg I/ml (diluted in incubation medium).

NRK 52-E cells were cultured on cell culture cover slips in thermanox plastic (NUNC, Denmark) in 24 well plates (Becton Dickinson, USA) until confluence was reached (normally 3 days). Cell media was removed and the cells were carefully washed in PBS before exposure to incubation medium and ICM. Incubation medium and ICM, 1 ml, was added to each well in duplicate and the plates were placed in the incubator during the exposure times. At the termination of the experiment, controls and ICM were removed by careful suction and the cover slip was washed in PBS followed by the immediate addition of 1 - 2 ml of 10 % neutral buffered formalin to fix the cells.

After a minimum of 24 hours fixation the formalin was removed from each well, and 1 - 2 ml of hematoxylin was added. After 1 hour the cover slips were removed, washed in sodium

chloride solution (9 mg/ml) and then left to air dry for 30 minutes. At the end of the drying period the cover slips were mounted onto glass microscope slides with mountant.

Necrotic and apoptotic cells were scored by light microscopic examination of hematoxylin stained sections at 600 times magnification. The morphological features used to identify and score necrotic and apoptotic cells have been described previously (Bursch et al., 1985;Levin, 1998;Levin et al., 1999;Mohr, 1999;Stephens et al., 1991). To determine the cell death index (CDI), five fields were selected from the two specimens (2 counts from one and 3 counts from the other). For each time point and in each field the number of viable cells and necrotic or apoptotic (dead or dying) cells were recorded.

The CDI is presented as a percentage of the total number of cells (± SEM). Each experiment was repeated two times, therefore no statistical analysis was preformed. A short description of the morphological features for all exposure periods is also given. All cell morphology evaluations were performed by a veterinary pathologist using light microscopy (Olympus BH-2 light microscope, Japan).

3.3.6 Internalization of iodinated x-ray contrast media

To test whether ICM are retained in the extracellular space or are internalized, NRK 52-E cells were exposed to ICM for different time periods. At the termination of the experiment, incubation medium (control) and ICM were decanted and cell extracts were prepared. Iodine content in the cell extracts were measured by Inductively Coupled Plasma Atomic Emission Spectrometry (ICP-AES) analysis, and protein content was measured by the Micro BCA™ Protein assay (Pierce, USA).

Iodinated x-ray contrast media exposure

NRK 52-E cells were exposed to incubation medium (control), iodixanol, iohexol, iopromide, and ioversol for 0.5, 1, 2, 3, 6, 12 and 24 hours. All ICM were tested at a concentration of 150 mg I/ml (diluted in incubation medium).

Confluent NRK 52-E cells (normally 3 days after sub cultivation) in 6 well plates (NUNC, Denmark) were washed carefully twice with PBS and exposed to incubation medium and ICM for different time periods. At the end of the exposure time, incubation medium and ICM

were removed, and the cells were washed three times with PBS to remove all ICM. Following washing, 1 ml 0.1% Triton X-100 in PBS was added to each well to dissolve the cells. The plates were incubated for 30 minutes at 37ºC (Termaks, Norway) in order for the cells to dissolve. Following this the cells were homogenised by pipetting and the cell solution were divided in two; one for ICP-AES analysis and one for protein assessment, and transferred to appropriate vials. The samples were frozen (- 20ºC) for later analysis. Each experiment was repeated five times.

Inductively Coupled Plasma Atomic Emission Spectrometry

ICP-AES analysis was preformed at the ICP lab at GE Healthcare (Analytical Sciences Research and Development). Iodine concentration in the samples was measured on ICP-AES analyser (Perkin Elmer Optima 3300DV ICP-AES with Perkin Elmer AS-91 Autosampler, USA). ICP-AES is a multi-element analysis technique that will dissociate a sample into its constituent atoms and ions, and cause them to emit light at a characteristic wavelength by exciting them to a higher energy level. This is accomplished by the use of an inductively coupled plasma source, usually argon. A monochromator can separate specific wavelengths of interest, and a detector is used to measures the intensity of the emitted light. This information is used to calculate the concentration of that particular element in the sample (Manning and Grow, 1997).

Briefly, cell extracts to be analysed were homogenised by pipetting, the samples (100 µl) were decomposed in 1 M ascorbic acid (100 µl), internal standard was added (500 µl of 10 µg Sc/ml), and the samples were further diluted in water to a total volume of 5 ml. All dilutions were preformed by an autodiluter (Hamilton Microlab 1000 autodiluter, USA). Blanks were prepared as the unknown samples (500 µl 10 µg Sc/ml, 100µl 1M ascorbic acid and 4400 µl water), and used to subtract the background iodine concentration. A calibration curve was prepared from different iodine concentrations (0, 5, 10 and 50 µg I/ ml) in 0.01 M ascorbic acid and 1 µg Sc/ml. Iodine concentrations in the samples were measured on ICP-AES analyser and the unknown samples were calculated from the standard curve.

Protein measurement

As the cell number in each well is not constant, and to compensate for dead and detached cells, protein measurements were preformed to have an indirect measurement of the cell density to correlate with the measured iodine concentration. Protein measurements for all the samples were analysed by the Micro BCA™ Protein assay (Pierce, USA).

The Micro BCA™ Protein assay utilizes bicinchonic acid (BCA) as the detection reagent for protein. The assay is mainly based on two reactions: (1) Cu^{+1} formes when Cu^{+2} is reduced by protein in an alkaline environment; (2) a purple coloured reaction product forms by the chelatation of two molecules of BCA with one cuprous ion (Cu^{+1}) (Smith et al., 1985). This water-soluble purple complex exhibits a strong absorbance at 562 nm.

The assay was conducted as described by the supplier. Briefly, 150 µl of each calibration sample and 150 µl of each unknown sample were transferred to separate dry and clean wells in a microtiter plate. Following this, 150 µl of the working reagent was added to each well, and the plate was then placed on a plate shaker for 30 seconds before incubated at 37 ºC for 2 hours. At the end of the incubation period, the plate was allowed to cool down to room temperature. When room temperature was reached, the plate was read at 562 nm (VERSAmax™ Turnable Microplate reader, Molecular Device, USA). The unknown samples were calculated from the calibration curve (SoftMax Pro 4.7.1., Molecular Device, USA). Bovine serum albumin was used as the protein to generate the calibration curve.

Calculation of the results

The results from the ICP-AES analysis (µg I/ml) were normalized to protein content (mg/ml) by the following formula; C_I / C_P, where C_I denotes iodine concentration in the cell extract and C_P denotes protein concentration in the cell extract.

3.4 *In vivo* and *ex vivo* methods

A pilot study and a definitive *in vivo* study were conducted to develop a rat model of renal impairment, and to elucidate potential biomarkers of renal damage caused by ICM. The animal model was used to induce renal impairment to simulate the patients at risk before ICM administration.

3.4.1 The rat-gentamicin pilot study

The pilot study was conducted to develop an improved rat-gentamicin model and to elucidate potential biomarkers after intravenous injection of ioversol (300 mg I/ml) in male and female rats.

12 rats (6 males and 6 females), 260 – 480 gram, were used in the pilot study. The study was conducted over two consecutive weeks to separate the males and the females. The rats were trained in metabolism cages for increasing time periods prior to experimental start. This was done to acclimatize the rats to metabolism cages and to minimize stress response bias in the study.

The rats were given gentamicin (Garamycin, Schering-Plough, USA) at a dose of 50mg/kg bw or saline sub-cutaneously (s.c.) for four consecutive days. On day 4 post injection, some of the rats were also given an intravenous (i.v.) dose of ioversol at 6 g I/kg (given at a rate of 0.6 ml/min). The study design is described in *Table 3*.

Table 3 Groups of animals in the Gentamicin Pilot study

Group	No of animals	Pre-treatment by sc injection (day 1-4)	Intravenous injection (day 4)
1	2 (1M + 1F)	Physiological saline	Physiological saline
2	2 (1M + 1F)	Physiological saline	Optiray
3	3 (1M + 2F)	Gentamicin	Physiological saline
4	3 (2M + 1F)	Gentamicin	Ioversol

Blood samples were taken from the animals three days before the study start and also on day five. Before blood sampling, the rat cages were placed on a warming mat, normally for 15 minutes, to facilitate the blood sampling. The rats were immobilized by wrapping them in a cloth and placing them on a custom built immobilization device. No anaesthesia was given. Their tail vein was venipunctured and approximately 1.3 ml blood was collected into a plain tube. The blood was allowed to clot for a minimum of 45 minutes, and serum was prepared from clotted blood by centrifugation at 2000 g for 10 minutes (Spinchron R Centrifuge, Beckman, USA), transferred to appropriate vials, and frozen (-70ºC) for later analysis.

Urine samples were taken three days before the study was to start and each day after s.c. injection. The samples were collected over 3 hours in metabolism cages (Tecniplast, Italy) placed on a urine refrigerated rack (Tecniplast, Italy). The urine was collected cold to avoid enzymatic and bacterial degradation of the urine. After collection, the urine samples were centrifuged at 1500 rpm to remove urinary sediment. Urine supernatants were transferred to appropriate vials and frozen (-20 or -70ºC) for later analysis of proteins and enzymes.

On the day of termination, day five, the rats were killed by an intravenous overdose of 25 mg/ml thiopental (4 ml/kg bw). The rats were opened by a mid-ventral incision of the abdomen and chest wall and the kidneys, brain, heart, thymus, liver, spleen, and lung/bronchi were taken out, examined for grossly visible changes, and immersion fixed in 10 % neutral buffered formalin.

Histopathology of the kidneys was performed as described in section 3.4.3. Clinical chemistry parameters (Serum; creatinine. Urine; creatinine, γ-glutamyl transferase (GGT), N-acetyl-β-D-glucosaminidase (NAG) and total protein) were analysed as described in section 3.4.5. α-Glutathione S-transferase (rat alpha GST EIA, Biotrin International, Dublin, Ireland) was measured in urine samples as described in section 3.4.6.

3.4.2 The rat-gentamicin model

To optimize the dose level of gentamicin, three different gentamicin dose groups were tested (50, 60 and 70 mg/kg bw, s.c. injection), and a physiological saline group was included as a control. A gentamicin-ioversol group was included in the study for evaluating biomarkers and to act as a comparator to the pilot study. The dose of gentamicin in the latter group was

decided based on the histopathology results of the dose-level experiments with gentamicin alone (50, 60 and 70 mg/kg bw).

Table 4 Study design

No of animals	Subcutaneous injection (day 1 -4)		Intravenous injection (day 4)	
	Compound	**Dose (mg/kg s.c.)**	**Compound**	**Dose (mg/kg i.v.)**
12 M	Physiological saline	-	-	-
12 M	Gentamicin	50	-	-
12 M	Gentamicin	60	-	-
12 M	Gentamicin	70	-	-
12 M	Gentamicin	Dose to be decided	Ioversol	20ml/kg (6 gI/kg)

The experiment was conducted over five consecutive weeks. The saline group and the gentamicin groups were divided in two, and animals from two different groups were handled every week. This was done to have the histopathology results ready to decide the definitive dose for the gentamicin-ioversol group before they were dosed the last week of the experiment, and because the urine refrigerated rack is limited to 12 metabolism cages.

All animals were acclimatized for 6 days before they were further trained in metabolism cages. The animals were trained for the urine sampling procedure in metabolism cages for 3 consecutive days before the baseline samples were taken. During the training period the rats were placed in metabolism cages once a day; 30 minutes the first day, 1 hour the next day and 3 hours the last day of the training period. In addition to this pre-training, the animals were placed in the metabolism cages again for re-training for 3 hours on day two of the study before the final urine samples were taken on day four.

Three days before the experiment was to start, a blood sample and a 12 hour urine sample was taken to obtain baseline control values for each animal (blood and urine samples were collected as described in the pilot study, section 3.4.1). A urine sediment sample was prepared from each centrifuged urine sample. The pellets, urine sediment consisting of epithelial cells, different casts and other wastes, were resuspended in the remaining urine (normally less than 100 µl) and 6 drops of 10 % neutral buffered formalin were added for fixation of the urine sediment. The fixed urine sediments were kept in the fridge until preparation (normally less

than 3 days).

On day one to four of the experiment the animals were given a s.c. injection of gentamicin (50, 60 or 70 mg/kg bw depending on the group) or physiological saline. On the days of dosing, the animals were weighed and the administered doses were calculated to the nearest 0.01 ml. The rats were immobilized by means of wrapping them in a cloth and holding them tight during the injections. No anaesthesia was given.

On the fourth day of dosing, after the gentamicin injection, the animals in the gentamicin-ioversol group were given ioversol (6 g I/kg) intravenously at a rate of 0.6 ml/min.

On day 4, urine samples were collected over 12 hours starting 1 hour after the final injection of gentamicin or ioversol (collected as described in the pilot study, section 3.4.1).

On day 5, blood samples were taken (collected as described in the pilot study, section 3.4.1), and after the necessary blood sampling the animals were killed by an intravenous overdose of 25 mg/ml thiopental (4 ml/kg bw). The rats were opened by a mid-ventral incision of the abdomen and the kidneys were taken out. The kidneys (both left and right) were examined for gross visible changes, weighed and fixed in 10 % neutral buffered formalin for a minimum of 24 hours before histopathological processing and examination.

Histopathology was decided to be the primary end-point of toxicity in the study, with a number of biomarkers being measured to gain further information on the model.

3.4.3 Histopathology

The formalin fixed-kidneys were processed by using standard methods previously described (Brayton et al., 2001;Relyea et al., 2000). Briefly, kidneys were trimmed into appropriate sizes (5 mm) (Microtome RM2155, Leica, Germany), water was removed from the tissue and the tissue was embedded in paraffin wax in a vacuum infiltration processor (Tissue-Tek® vacuum infiltration processor, Sakura, USA). The tissue paraffin blocks was sectioned at a normal thickness of 3 -5 µm by a microtome (RM2155, Leica, Germany). Tissue wax sections were further transferred to a water bath which allows the sections to be stretched out, followed by transfer to a glass slide and incubation in a drying cabinet for the paraffin wax to melt (1 hour at 80º C). The sections were warmed in histo-clear® II solution, and then hydrated through graded alcohols to water. Following this the sections were stained with hematoxylin and eosin in an autostainer (DRS-60, Sakura, USA). The sections were dehydrated rapidly through graded alcohols, and finally cleared in histo-clear® II solution.

The slides were taken from the last clearing bath and a mounting medium (SUB-X mounting medium, Surgi-Path, UK) was placed onto the section followed by covering with a glass cover slip. All tissues were examined by a veterinary pathologist using light microscopy (Olympus BH-2 light microscope, Japan).

3.4.4 Urine Cytology

Quantitative cytology analysis

For quantitative cytologic analysis, 20 µl of 1 % eosin solution diluted in phosphate buffered saline was added to 0.5 ml of urine and mixed well. Next, the urine sample was loaded into a Bürkers chamber and the number of epithelial cells was counted. The number of epithelial cells (Ep) excreted per minute was then calculated with the following formula:

$$Ep/min = (X \times V) / (t \times V_B)$$

where X = number of epithelial cells in 100 fields, V = volume of urine (ml), t= duration of collection of urine (min), and V_B = volume of 100 viewing fields.

Morphological analysis

Well-mixed urine, 200µl, was cytocentrifuged at 750 rpm (65 x g). After cytocentrifugation 1 -2 drops of Parlodion adhesive was placed over the cells on the slide, and then the slide was fixed in Saccommano's fixative for 10 minutes. After fixation, a rapid progressive Papanicolaou staining method was applied: the slide was placed in distilled water for 1 minute, Gill 2 hematoxylin for 7 – 10 minutes, distilled water for 30 seconds, 95% ethanol for 1 minute, OG-6 for 1 minute, 95% ethanol for 20 seconds, EA-50 for 1.5 minutes, 95% ethanol for 1.5 minutes, 100% ethanol for 3 minutes, and finally cleared in xylene for 3 minutes.

After staining the slides were coverslipped. Cytological evaluation of the cells was made using the morphological criteria suggested by Schumann (1985) to assess the numbers of convoluted renal tubule, collecting duct, and necrotic renal tubule cells. An evaluation of the total number of renal tubule cells was made to generate a urinary cytodiagnosis based on the classification of the findings by Schumann (1985). The preparations were done at the Veterinarian Institute (Oslo), and all preparations were examined by a veterinary pathologist using light microscopy (Olympus BH-2 light microscope, Japan).

3.4.5 Clinical chemistry

Clinical chemistry was assessed on a Beckman Synchron CX® System (USA) at GE Healthcare. Samples were loaded into racks in appropriate dilution, and following this, the racks were placed in the machine. From there on, the machine takes care of all the dilutions, mixing of samples and reagents, and calculation of the results.

Creatinine

A creatinine reagent was used to measure the creatinine concentration by using a modified Jaffé method. In the reaction, creatinine combines with picrate in an alkaline solution and forms a creatinine-picrate complex. The absorbance at 520 nm is monitored by the system and used to calculate the creatinine concentration (mmol/l for urine and µmol/l for serum) in the sample.

Total protein

Total protein content in the samples was measured by a modified micro pyrogallol red procedure. The method is based upon measuring the shift in the red absorption that occurs when the pyrogallol red-molybdate complex binds to basic amino acid groups of protein molecules. The increase in absorbance at 600 nm is directly proportional to the protein consecration in the sample (mg/dl).

N-acetyl-β-D-glucosaminidase

A NAG reagent was used to measure NAG activity in the urine samples. The principle of the method is that 3-Creosolsulfonphthaleinyl-N-acetyl-β-D-glucosaminide, a sodium salt, is hydrolysed by NAG resulting in release of 3-creosol-sulfonphthalein. The absorbance at 580 nm is monitored by the system and used to calculate the NAG activity (IU/ml).

γ-Glutamyl transferase

A GGT reagent was used to measure GGT activity in the urine samples. GGT catalyses the transfer of a γ-glutamyl group from the colorless substrate, γ-glutamyl-p-nitroaniline, to the acceptor, glycylglycine, and the product is p-nitroaniline. The absorbance at 410 nm is monitored by the system and used to calculate the GGT activity (IU/ml) in the samples.

3.4.6 α-Glutathione S-transferase enzyme immunoassay

The level of α-glutathione S-transferase (α-GST) in urine samples was assessed by a rat α-GST EIA (Biotrin International, Ireland). The test procedure is based on sequential addition of sample, antibody-enzyme conjugate, and substrate to microassay wells coated with anti rat α-GST IgG. The resultant colour intensity is proportional to the amount of α-GST in the sample.

A wash solution, calibrators, and positive controls were prepared as described in the assay protocol, and urine samples were diluted as recommended by supplier (5 times dilution). The calibrators (in duplicate), the positive control (in duplicate) and the samples (single), 100 µl of each, were transferred to the microassay plate. The plate was covered and incubated in an incubator at 25 ºC for 60 minutes with uniform shaking. At the end of incubation, the plate was washed 6 times with a wash solution in a microassay strip washing system (Multi Wash, Tri-continent scientific, USA). The plate was firmly tapped against paper to remove all washing buffer. Following this, 100 µl conjugate solution was added to each well, and the plate was incubated in an incubator at 25 ºC with uniform shaking for 60 minutes. At the end of the incubation time, the plate was washed as previously described. Substrate solution, 100µl, was added to each well and the plate was incubated at room temperature for exactly 15 minutes before 100 µl stop solution was added to each well. The plate was immediately read at 450 nm with 630 nm as a reference (VERSAmax™ Turnable Microplate reader, Molecular Device, USA). Mean absorbance was calculated for each calibrator and positive control. A calibration curve was plotted with the absorbance at 450-630nm versus the α-GST concentration (µg/l) indicated by the mean absorbance of the calibrators.
The unknown samples were read from the calibration curve (SoftMax Pro 4.7.1., Molecular Device, USA) and multiplied with the appropriate dilution factor to generate the true α-GST concentration in the samples.

3.4.7 Cystatin C enzyme linked immunosorbent assay

Cystatin C in both serum and urine was measured on a human ELISA kit (Alexis Biochemicals, USA) (cross reactivity between species are reported). The assay is based on sequential addition of sample, antibody-enzyme conjugate, and substrate to microassay wells coated with polyclonal anti human cystatin C specific antibody.

Wash solution, conjugate solution, dilution buffer, calibrators and quality controls (high and low) were prepared as described in the assay protocol. Both urine samples and serum samples were assessed at the lowest possible dilution (4 times- for serum and 20 times dilution for urine). Calibrators (in duplicate), quality controls (in duplicate), and samples (single) (100 µl) were transferred to the wells of the plate. The plate was incubated in an incubator at 26 ºC for 30 minutes with uniform shaking. Any cystatin C present in the sample is captured and immobilized by cystatin C antibody in the well. At the end of the first incubation period, the wells were washed 3 times with 350 µl of wash solution in a microassay strip washing system (Multi Wash, Tri-continent scientific, California, USA). Any unbound protein was washed away. The plate was tapped firmly on paper to remove any remaining wash solution. Horseradish peroxidase (HRP) conjugated polyclonal anti human cystatin C antibody was added to all wells (100 µl) and the plate was incubated in an incubator at 26 ºC with uniform shaking for another 30 minutes. Following another wash step (as previously described) to remove unbound antibody HRP-conjugate, 100 µl substrate solution (H_2O_2 and TMB) was added to all wells and the plate was incubated for 10 minutes at room temperature without shaking. At the end of the exposure time 100 µl of an acidic stop solution was added to each well, and the plate was immediately read at 450 nm (VERSAmax™ Turnable Microplate reader, Molecular Device, USA).

The intensity of the colour, measured spectrophotochemically at 450 nm, is directly proportional to the amount of cystatin C bound in the initial step. Concentrations of the unknown samples were read from the standard curve that was constructed by plotting the absorbance values against each human cystatin C standard level using a four-parameter function (SoftMax Pro 4.7.1., Molecular Device, USA).

3.4.8 Calculations of urine parameters

Urinary excretion of NAG, GGT, and α-GST was calculated by the formula U_p/U_{Cr}, where U_p denotes urinary concentration of each of the parameters mentioned above and U_{Cr} urinary concentration of creatinine. Creatinine and total protein was calculated as total excretion over the 12 hour collection period.

3.5 Statistical analysis

The statistical analyses were preformed in SigmaStat version 3.1 and GraphPad Prism version 4.0, and both programs were used to make illustrations.

Extreme values and outliers were omitted from the statistical analysis and illustrations. Outliers were defined as $< Q_1 - 1.5$ IQR (inter quartile range) and $> Q_3 + 1.5$ IQR.

The Kolmogorov-Smirnov test was used to test for normal distribution, and homogeneity of variance was tested using the Levene's test. Normally distributed results with homogeneity of variance were analysed with one-way ANOVA. Where statistically significant differences were found, Dunnet's test was used to compare treatment groups against a control group, and when assessment of differences between the different treatments groups was needed, Duncan's test was used.

Where non-homogeneity of variance or non-normal distributions was found the non-parametric Kruskal-Wallis ANOVA on ranks was applied, and the post-hoc testing was done by using the Dunn's test.

The results are expressed as mean ± SEM throughout the result chapter, with the exception of a few tables where standard deviation was also included. Significance level was set to $p < 0.05$.

4. Results

4.1 *In vitro* results

4.1.1 Cell death assesed by the trypan blue exclusion assay

Assessment of cell death by the trypan blue exclusion assay after exposure to ICM over different time periods revealed a marked difference in cell death. *Figure 5* and *Table 5* show how cell death increased in a time dependent manner, and how the four ICM differ in their potential to induce cell death.

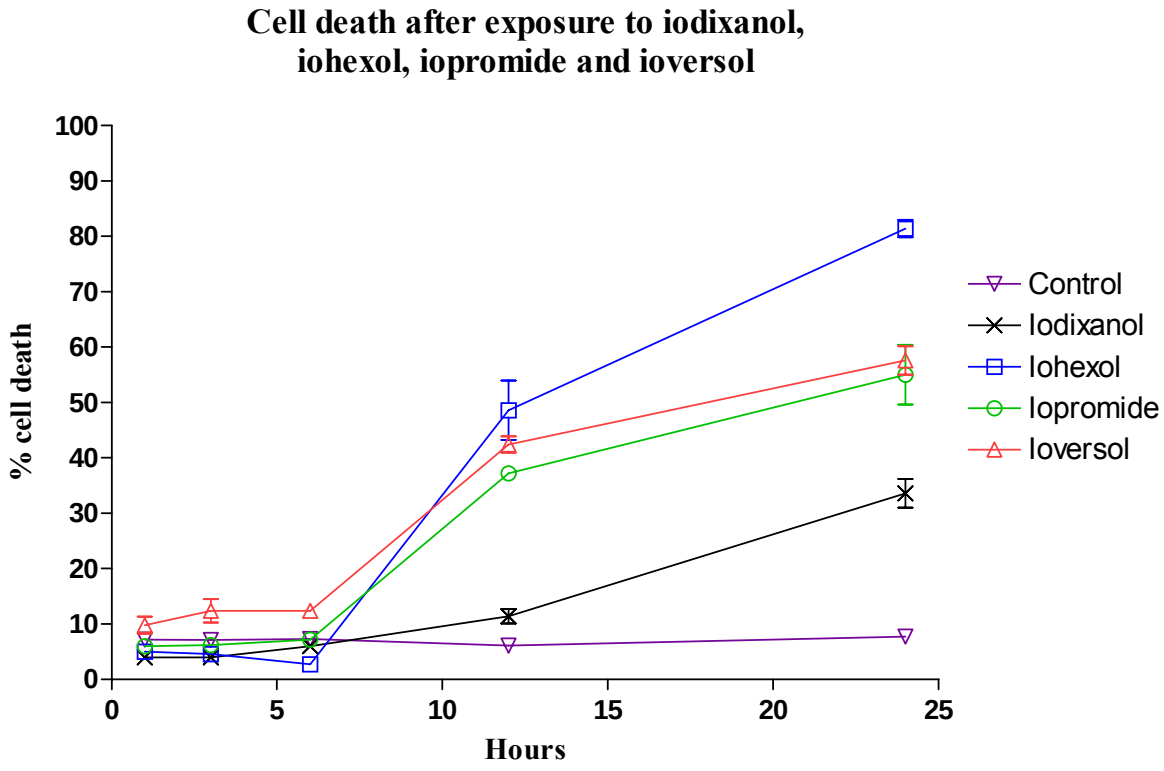


Figure 5 Percent cell death measured by trypan blue exclusion assay in NRK 52-E cells at 1, 3, 6, 12 and 24 hours exposure to incubation medium (control), iodixanol, iohexol, iopromide and ioversol (150 mg I/ml). Mean ± SEM are plotted. 4 ≤ n ≤ 5 for ICM groups and n = 20 for control (one control for each ICM counted).

Table 5 Percent cell death measured by the trypan blue exclusion assay in NRK 52-E cells after exposure to incubation medium (control), iodixanol, iohexol, iopromide and ioversol (150 mg I/ml)

	Percent cell death (mean ± SEM)				
	1 hour	3 hours	6 hours	12 hours	24 hours
Control (I)	7.20 ± 0.51	7.15 ± 0.65	7.20 ± 0.63	6,10 ± 0.26	7.72 ± 0.56
Iodixanol (II)	4.00 ± 0.32 *	4.00 ± 0.32	6.00 ± 1.00	11.40 ± 1.29	33.60 ± 2.58 *
Iohexol (III)	5.00 ± 0.89	4.60 ± 0.68	2.75 ± 0.25 *	48.60 ± 5.38 *	81.40 ± 1.57 *
Iopromide (IV)	6.00 ± 0.55	6.20 ± 0.74	7.20 ± 0.37	37.20 ± 0.86 *	55.00 ± 5.40 *
Ioversol (V)	9.80 ± 1.53	12.40 ± 2.11*	12.40 ± 1.25 *	42.40 ± 1.47 *	57.70 ± 2.62 *
Significance**	II vs. V	II vs. V	II vs. V	II vs. III	II vs. III
	III vs. V	III vs. V	III vs. IV	II vs. IV	II vs. IV
	IV vs. V	IV vs. V	III vs. V	II vs. V	II vs. V
			IV vs. V	III vs. IV	III vs. IV
				III vs. V	III vs. V

** indicates statistically significant difference relative to control (one way ANOVA with Dunnet's post hoc test, 4 ≤ n ≤ 5 for the ICM groups and 20 for the control group, p < 0.05). ** indicated statistically significant difference in pairwise comparison (Duncan's method, p < 0.05).*

The total data set was considered to be of normal distribution and with homogenous variance as the majority of the experiments fell within the criteria for normal distribution and homogenous variance (see discussion for details).

At early time points (up to 6 hours exposure) the cell death in the ICM groups was similar to the control groups. After 6 hours exposure this was changed, and the cell death caused by ICM became more pronounced compared to the controls. At 12 and 24 hour exposure iohexol caused the most pronounced cell death of the four ICM (34 % and 81 %, respectively), and at both time points the cell death was statistically significantly higher than the control and the other ICM. Ioversol caused a slightly higher cell death than iopromide at 12 and 24 hour exposure time (42 % and 58 % vs. 37 % and 55 %, respectively). They both induce a statistically significantly higher cell death than the control. However, no statistical significant difference was detected when they were compared pairwise. Iodixanol caused the least pronounced cell death of the four ICM at 12 and 24 hour exposure (11 % and 37 %). At 12 hour exposure, the cell death caused by iodixanol was not statistically significantly different from the control, however it was statistically significantly lower than the other ICM. At 24

hour exposure, the cell death induced by iodixanol was statistically significantly higher than the control and statistically significantly lower when compared pairwise with the other ICM.

To test the relationship between cell death and osmolality, a linear regression analysis was performed at 24 hour exposure. The linear regression analysis, illustrated in *Figure 6*, show that there is a relatively good relationship between the induced cell death and the osmolality of the ICM.

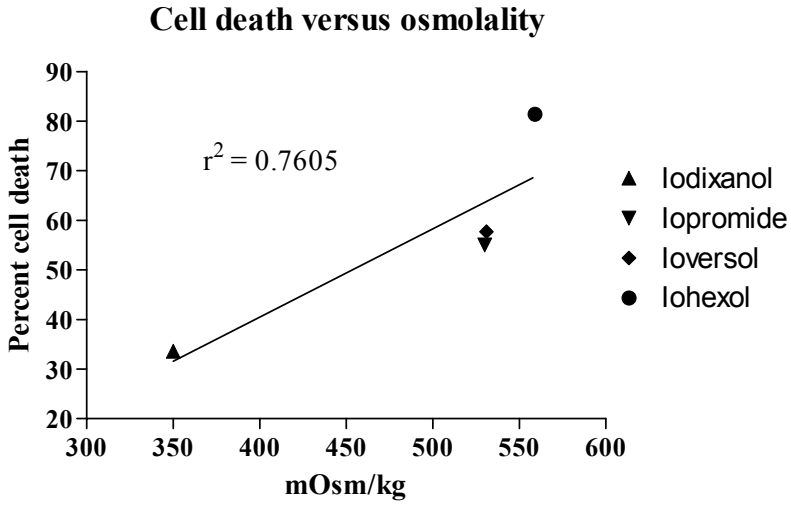


Figure 6 Scatterplot of percent cell death at 24 hour exposure to iodixanol, iohexol, iopromide, and ioversol (150 mg I/ml) versus osmolality with a linear regression line. Mean cell death are plotted.

Iohexol has the highest osmolality of the ICM, followed by ioversol, iopromide, and iodixanol. The results from the trypan blue exclusion assay indicate that the cell death appeared to be correlated to the osmolality of ICM, as the cell death increase with increasing osmolality. Furthermore, the three LOCM showed a marked difference in their potential to induce cell death, indicating that the chemical structure of the contrast agents may partly be related to the toxicity.

4.1.2 Cell viability assessed by the MTT assay

The results from the MTT assay show that iodixanol, iohexol, iopromide, and ioversol inhibited mitochondrial dehydrogenase activity, as determined by MTT reduction, compared to the control (100 % at all exposure times). *Figure 7* and *Table 6* show how the four ICM decreased MTT conversion in a time dependent manner and how they differ in their potential to inhibit MTT reduction.

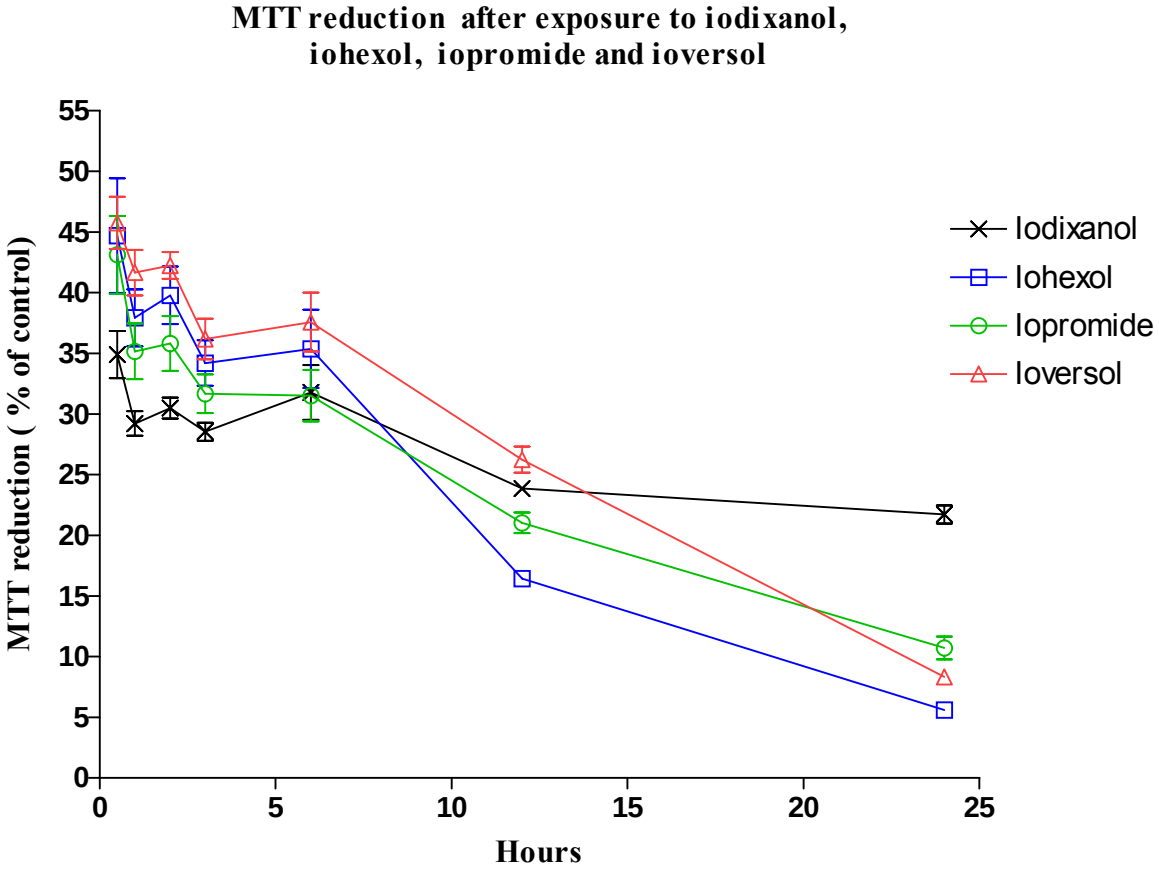


Figure 7 MTT reduction measured by the MTT assay on NRK 52-E cells at 0.5, 1, 2, 3, 6, 12 and 24 hours exposure to iodixanol, iohexol, iopromide, and ioversol (150 mg I/ml). Mean ± SEM are plotted. 5 ≤ n ≤ 8.

Table 6 Cell viability, as determined by mitochondrial dehydrogenase activity, in NRK 52-E cells after exposure to incubation medium (control), iodixanol, iohexol, iopromide, and ioversol (150 mg I/ml)

	Cell viability (% of control) as determined by mitochondrial dehydrogenase activity (mean ± SEM)						
	0.5 hour	1 hour	2 hours	3 hours	6 hours	12 hours	24 hours
Control (I)	100.00 ± 0	100.00 ± 0	100.00 ± 0	100.00 ± 0	100.00 ± 0	100.00 ± 0	100.00 ± 0
Iodixanol (II)	34.91 ± 1.94	29.22 ± 1.01	30.50 ± 0.87	28.54 ± 0.74	31.76 ± 2.26	23.87 ± 0.56	21.71 ± 0.76
Iohexol (III)	44.69 ± 4.73	37.92 ± 2.35	39.77 ± 2.37	34.21 ± 1.87	35.36 ± 3.22	16.42 ± 0.57	5.59 ± 0.37
Iopromide (IV)	43.14 ± 3.23	35.18 ± 2.28	35.82 ± 2.24	31.64 ± 1.60	31.52 ± 2.12	21.02 ± 0.85	10.73 ± 0.94
Ioversol (V)	45.75 ± 2.15	41.67 ± 187	42.22 ± 1.10	36.15 ± 1.68	37.58 ± 2.43	26.21 ± 1.08	8.32 ± 0.33
Significance *		II vs. III	II vs. III	II vs. III		II vs. III	II vs. III
		II vs. IV	II vs. IV	II vs. V		II vs. IV	II vs. IV
		II vs. V	II vs. V			II vs. V	II vs. V
		IV vs. V	IV vs. V			III vs. IV	III vs. IV
						III vs. V	III vs. V
						IV vs. V	IV vs. V

** indicates statistically significant difference in pairwise comparison (Duncan's method, p < 0. 05).*

Already at early time points the MTT conversion by mitochondrial dehydrogenases is greatly reduced (35 % to 46 % of the control). The MTT conversion is further reduced as the exposure time increases. All four ICM tested inhibited the mitochondrial dehydrogenase activity significantly more than the control (considered to induce no inhibition) at all exposure times.

From 0.5 to 6 hour exposure time, the reduction in MTT conversion is fairly stable, and the four ICM does not differ much in their potential to inhibit mitochondrial dehydrogenase activity. At 12 and 24 hour exposure, the reduction in MTT conversion is further reduced and the difference in potential of the four ICM to inhibit mitochondrial dehydrogenase activity becomes more pronounced.
Iohexol showed the strongest reduction of the MTT conversion at 12 and 24 hour exposure (16 % and 6 %, respectively). At both exposure times, the inhibition of mitochondrial dehydrogenase activity caused by iohexol was statistically significantly higher compared to the other ICM. Iopromide and ioversol reduced the MTT conversion to a slightly less degree than iohexol at 24 hours (11 % and 8 % versus 6 %, respectively). The reduction in MTT conversion caused by the two showed to be statistically significantly different when compared pairwise, and both MTT conversion reductions was statistically significantly lower than iohexol. Even though iohexol, iopromide, and ioversol proved to be statistically significant from each other in inhibiting mitochondrial dehydrogenase activity, their inhibitory potential did not differ much in percent (6 %, 11 % and 8 %, respectively). Iodixanol induced the least pronounced reduction in MTT conversion of the four ICM tested. After 12 hour exposure, the reduction of the MTT conversion caused by iodixanol seems to stabilise. At the 24 hour exposure time point, the MTT conversion was reduced to approximately 22 %, statistically significantly less reduced than the other ICM.

To test the relationship between the reduced cell viability and osmolality, a linear regression analysis was performed on cell viability after 24 hours exposure. *Figure 8* show that there is a strong relationship between the reduced viability and the osmolality of the ICM.

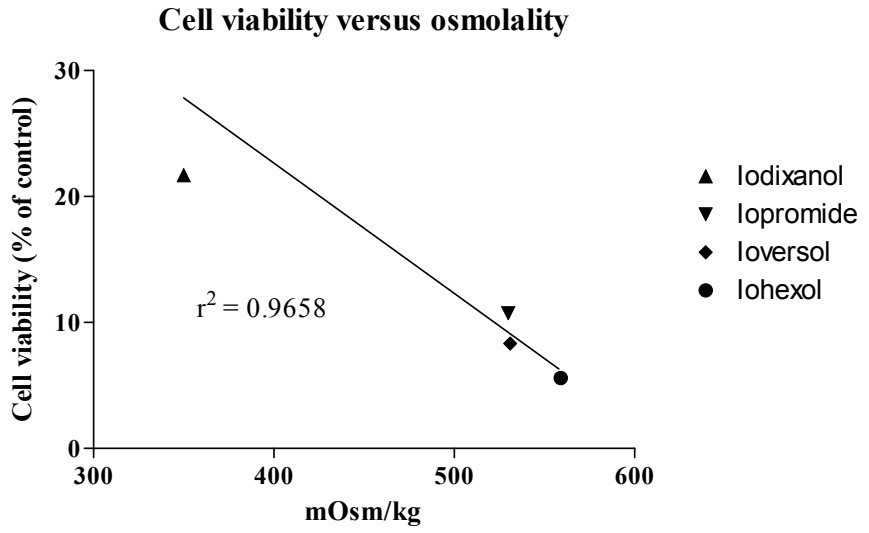


Figure 8 Scatterplot of cell viability at 24 hour exposure to iodixanol, iohexol, iopromide, and ioversol (150 mg I/ml) versus osmolality with a linear regression line. Mean cell viability (% of control) are plotted

These results indicate that the osmolality may be responsible for the inhibition of mitochondrial dehydrogenase activity, as the MTT conversion was reduced with increasing osmolality.

4.1.3 Cell morphology

Cell death assessment of formalin fixed NRK 52-E cells

The results from cell death assessment on the formalin fixed NRK 52-E cells after exposure to iodixanol, iohexol, iopromide, and ioversol over different time periods show that they all induced a pronounced cell death. *Figure 9* and *Table 7* show how the four ICM induce cell death in a time dependent manner and how they differ in their potential to induce cell death.

Since each experiment was conducted with only two replicates, no statistical analysis will be presented here. The n = 5 that is listed is the number of cell counts (pseudo-replicates), two counts from the first specimen and three from the last.

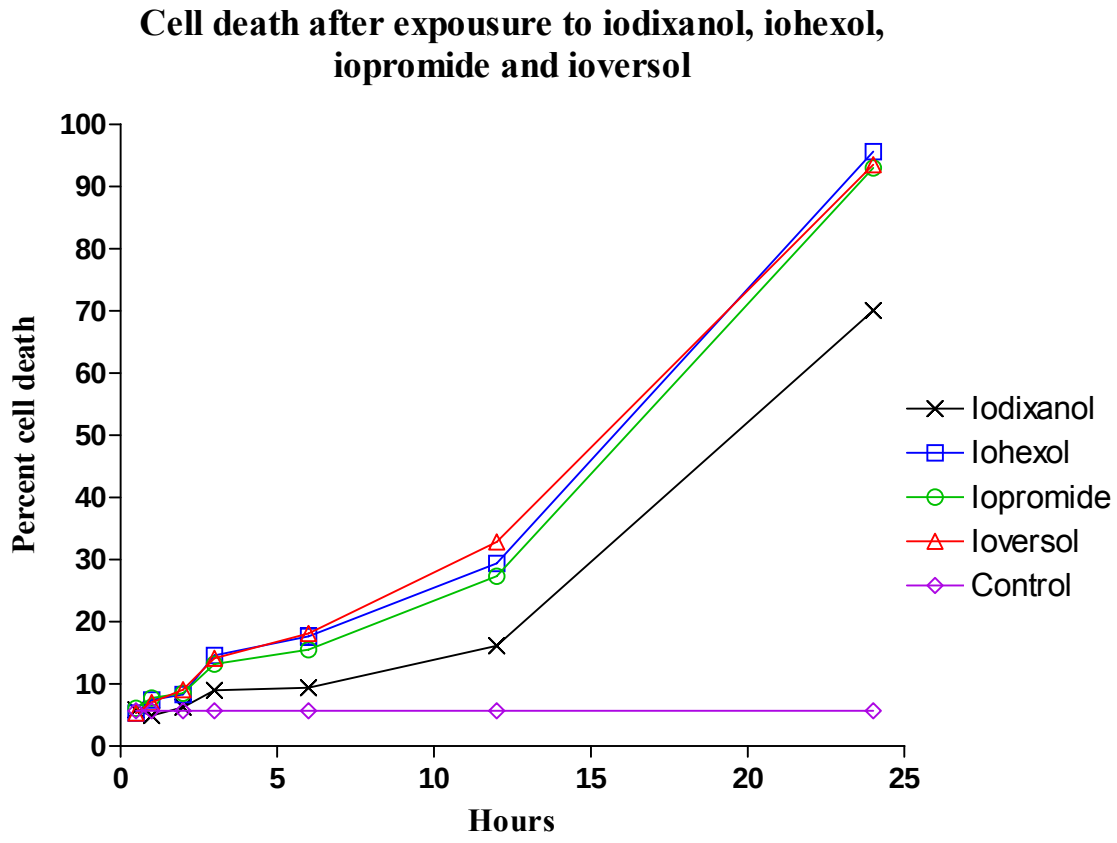


Figure 9 Assessment of cell death on formalin fixed NRK 52-E cells at 0.5, 1, 2, 3, 6, 12 and 24 hours exposure to incubation medium (control), iodixanol, iohexol, iopromide, and ioversol (150 mg I/ml). The control is the mean of all controls counted. Mean ± SEM are plotted. n = 5.

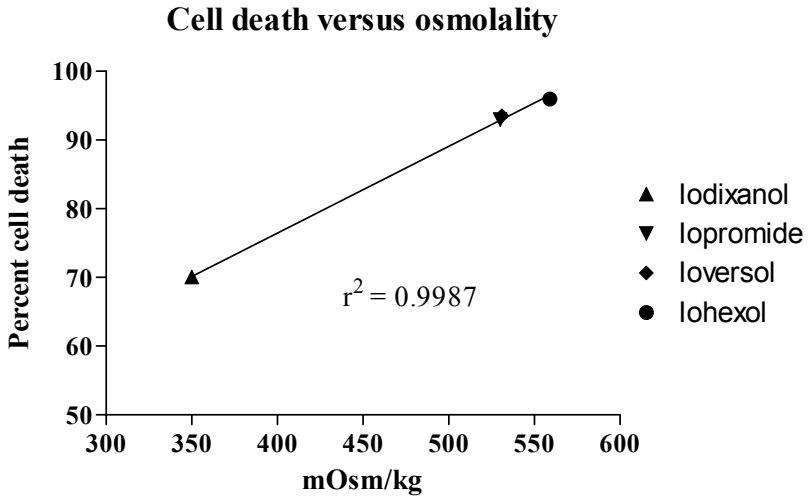


Figure 10 Scatterplot of cell death at 24 hour exposure to iodixanol, iohexol, iopromide, and ioversol (150 mg I/ml) versus osmolality with a linear regression line. Mean cell death are plotted.

Table 7 Percent cell death assessed on formalin fixed NRK 52-E cells after exposure to incubation medium (control), iodixanol, iohexol, iopromide, and ioversol (of 150 mg I/ml) (n = 5)

	Percent cell death (mean ± SEM)							
	Control	0.5 hour	1 hour	2 hours	3 hours	6 hours	12 hours	24 hours
Iodixanol	5.76 ± 0.55	5.83 ± 0.39	4.94 ± 0.28	6.26 ± 0.16	9.00 ± 0.37	9.40 ± 0.65	16.16 ± 0.71	70.05 ± 0.70
Iohexol	5.70 ± 0.42	5.33 ± 0.54	7.44 ± 0.33	8.32 ± 0.44	14.60 ± 0.38	17.64 ± 1.34	29.41 ± 0.25	95.59 ± 0.70
Iopromide	5.97 ± 0.42	6.11 ± 0.38	7.75 ± 0.39	8.52 ± 0.46	13.20 ± 0.24	15.52 ± 0.50	27.32 ± 1.15	92.99 ± 0.85
Ioversol	5.43 ± 0.73	5.34 ± 0.30	7.02 ± 0.41	9.09 ± 0.54	14.13 ± 1.00	18.09 ± 0.83	32.83 ± 1.07	93.51 ± 0.67

The results show that the cell death caused by the four ICM increased already after 3 hours exposure, and that the cell death increased further as the exposure time increases. Iodixanol induced the least pronounced cell death at most time points, and the difference between iodixanol and the other ICM is the most pronounced at 6, 12, and 24 hour exposure. Iohexol, iopromide, and ioversol seem to induce about the same degree of cell death at all exposure times. To test the relationship between reduced cell death and osmolality, a linear regression analysis was performed on cell death after 24 hours exposure to the four ICM versus osmolality of the ICM. The analysis, illustrated in *Figure 10*, show that there is a strong relationship between them.

Overall, the results suggest that the cell death induced by ICM can be attributed to the osmolality, as the cell death increased with increasing osmolality and because of the strong relationship between cell death and osmolality.

Cell features

The cells from all ICM groups at the 0.5, 1 and 2 hour time points appeared to have normal morphology when compared with the control (incubation media). The cells showed distinct cell borders, moderate amount of cytoplasm, and a large nuclei that contains coarsely stippled chromatin and often 1 or 2 nucleoli. At the 3 hour time point, the cells exposed to iohexol, iopromide and ioversol had numerous individual dead cells scattered throughout the slide. The dead cells showed condensed or fragmented nuclei, and the cytoplasm had contracted or the cell border was not distinguishable and was not in contact with the surrounding cells. At the 3 hour time point, the cell death after exposure to iodixanol was similar to the control, however numerous small clear round vacuoles were noted in the cytoplasm of the cells. At the 6 hour time point, the cells exposed to iohexol, iopromide and ioversol showed an increase in cell death and a loss of confluence of the cell layer. Also within the cytoplasm in the iohexol, iopromide and ioversol treated cells there were numerous small clear round vacuoles. The iohexol treated cells contained vacuoles of a similar number, but were of a smaller size than that seen in the iodixanol treatment group, whereas the ioversol and iopromide treated cells contain fewer and smaller vacuoles when compared with the cells in the iodixanol treatment group. The cells exposed to iodixanol for 6 hours showed less cells death than cells exposed to the other ICM, however the size and number of vacuoles was greater than in the cells exposed to the other ICM and the cells exposed to iodixanol for 3 hours. At the 12 hour time point, the amount of cell death and loss of confluence had increased in all ICM groups

compared to the 6 hour exposure and the control. The degree of vacuolation was similar to the 6 hour time point. At the 24 hour time point in all the ICM groups, there was extensive cell death, and a total loss of confluence. The vacuolation was not observable in the dead cells. In the viable cells exposed to iodixanol the degree of vacuolation was similar to that at the 3 hour time point.

Figure 11 a – d show representative pictures of the cell features after exposure to ICM.

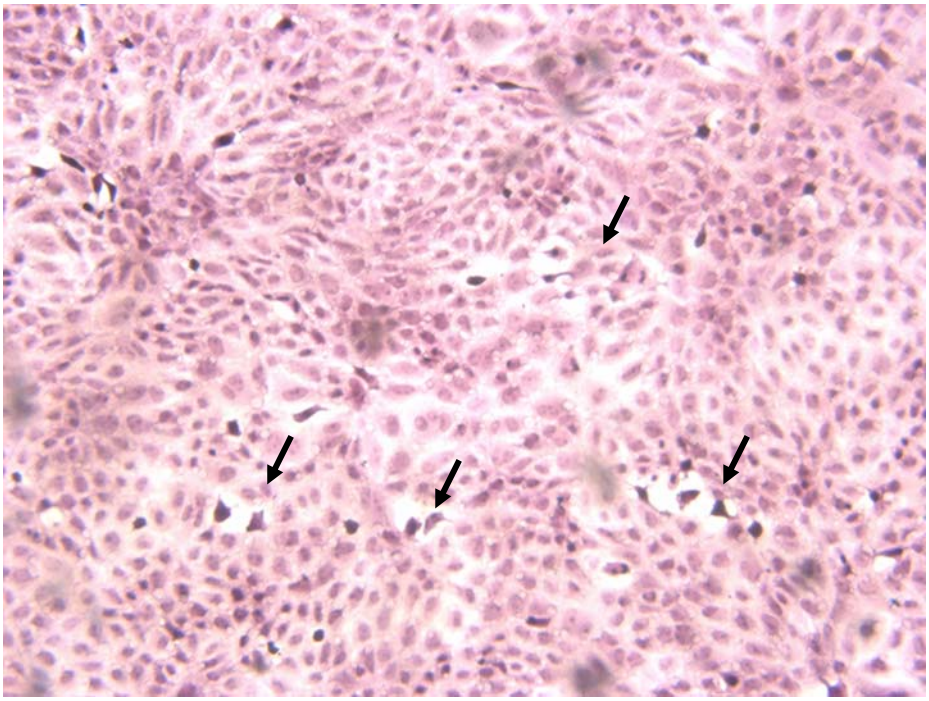


Figure 11 a. Hematoxylin stained NKR 52-E cells (control - incubation media treated) (x200). Note confluence of the cell layer. Randomly scattered throughout the culture are cells that have separated from the surrounding cells and contain condense dark nuclei (arrows). These are degenerated/dying cells (natural turnover).

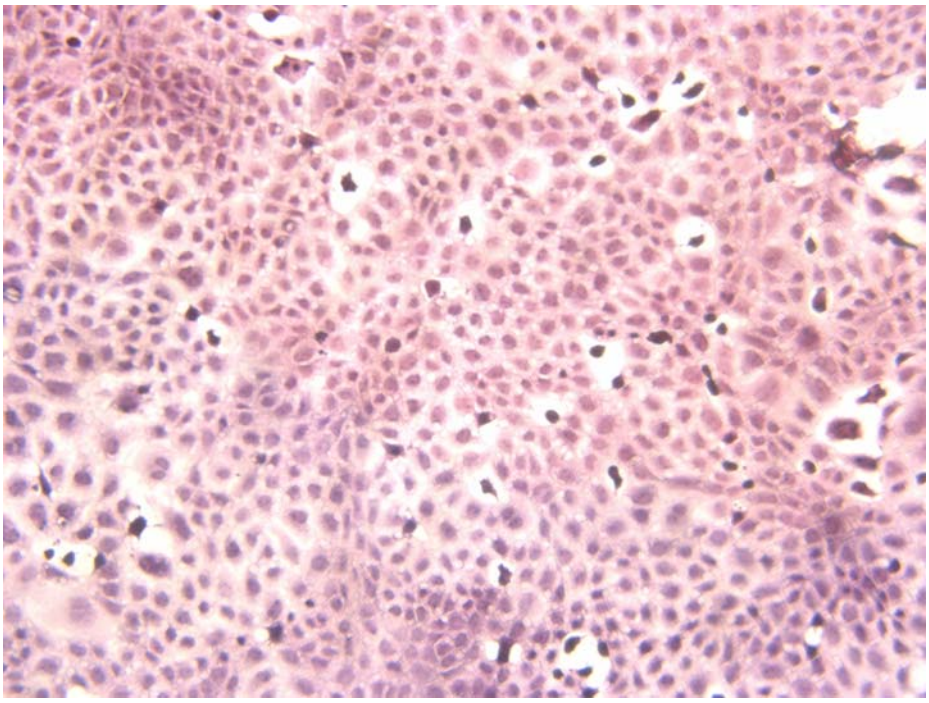


Figure 11 b. Hematoxylin stained NRK 52-E cells after 1 hour exposure to 150 mg I/ml ioversol (x400). Note the increased number of dead cells scattered throughout the cell culture layer.

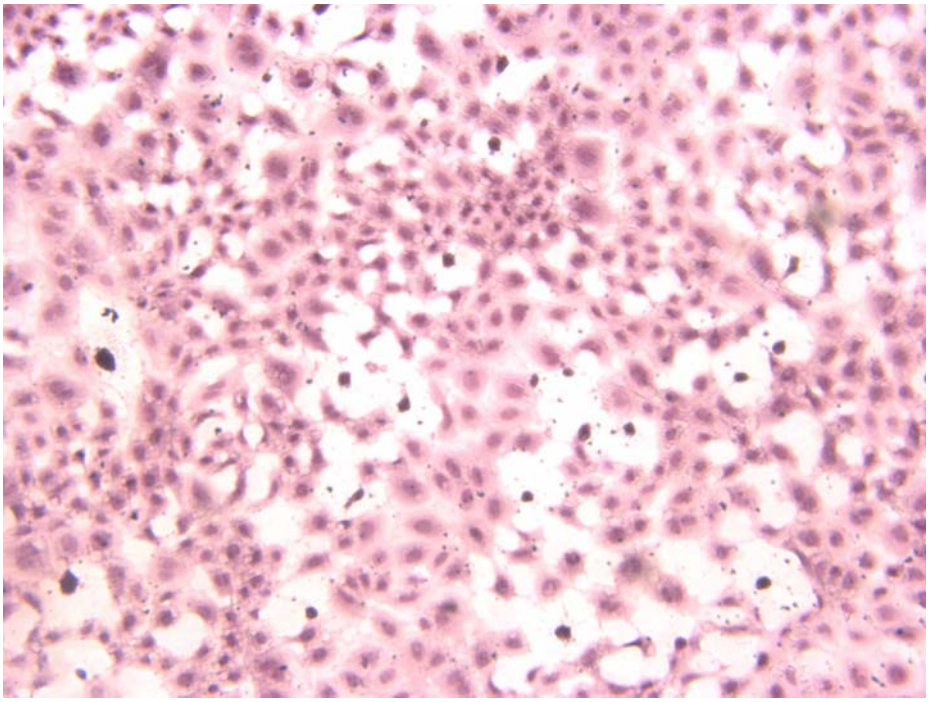


Figure 11 c. Hematoxylin stained NRK 52-E cells after 3 hours exposure to 150 mg I/ml ioversol (X400). There are more necrotic cells and the confluence of the cell culture layer is lost.

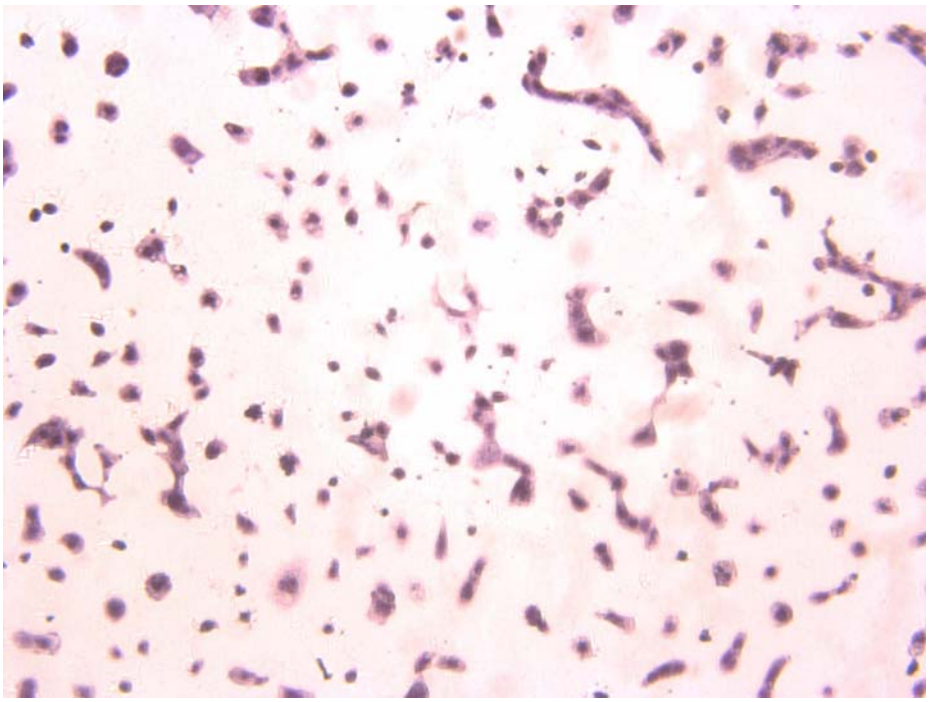


Figure 11 d. Hematoxylin stained NRK 52-E cells after 12 hours exposure to 150 mg I/ml ioversol (X400). Note that almost all the cells are either necrotic or dying. Also cell-to-cell contact is minimal.

4.1.4 Internalization of iodinated x-ray contrast media

The results from ICP-AES analysis normalized to protein content showed that there is a time dependent increase in internalization of ICM, that the internalization of the four ICM differ, and that the variation between the five replicates were high.

Table 8 Internalization (µg I/mg protein) of iodixanol, iohexol, iopromide, and ioversol in NRK 52-E cells

	Iodixanol	Iohexol	Iopromide	Ioversol
Exposure time	Mean ± SEM	Mean ± SEM	Mean ± SEM	Mean ± SEM
0.5 hour	523 ± 69	386 ± 82	498 ± 115	403 ± 126
1 hour	315 ± 38	311(± 20	400 (± 37)	496 ± 29
2 hours	744 ± 109	958 ± 401	750 ± 151	670 ± 168
3 hours	463 ± 126	541 ± 151	535 ± 161	458 ± 103
6 hours	607 ± 97	715 ± 95	699 ± 139	866 ± 264
12 hours	974 ± 185	1488 ± 249	1738 ± 448	1717 ± 285
24 hours	1237 ± 295	4443 ± 1251	2616 ± 1131	3861 ± 1149

Internalization was measured by ICP-AES analysis on cell extracts and normalized to protein content measured by the BCA protein assay. $4 \leq n \leq 5$.

As expected, the iodine concentration in the treated cells was higher than the control (incubation media) (0 µg I/mg protein) at all exposure time points for all the ICM. To evaluate if there was any differences between the ICM treatments, iodine content in all cell extracts was further compared pairwise at the same exposure time.

All time points passed the normality and equal variance test. The only detected statistically significant difference (one way ANOVA, Duncan's post hoc test, $p > 0.05$) was after 1 hour exposure; iodine content in cell extracts exposed to ioversol showed to be statistically significantly higher than the iodine content in cell extracts exposed to iodixanol and iohexol.

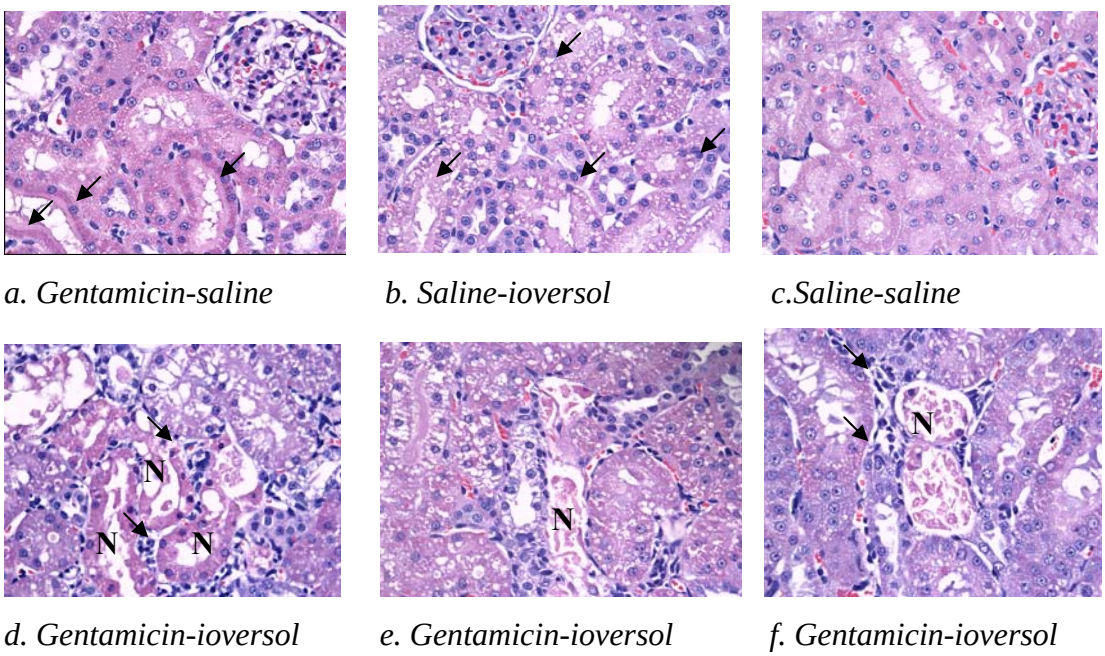
Even though no statistically significant differences were detected between the four ICM at most exposure times, it is possible to see a trend in the data. Uptake of ICM tends to increase with exposure time, with increasing osmolality, and/or with increasing cell death or reduced cell viability. At early time points, up to 6 hours, the four ICM seem to be taken up in the cells to a similar degree. At the 12 and 24 hour time point, the differences were more pronounced. Iohexol was most extensively internalized ICM at 24 hour, followed by ioversol, iopromide, and iodixanol.

The internalization data of ICM in NRK 52-E cells indicate that uptake of ICM may be correlated to the osmolality, as the internalization increases with increasing osmolality. Furthermore, the uptake of ICM could possibly contribute to reduced cell viability and cell death.

4.2 *In vivo* and *ex vivo* results

4.2.1 The rat-gentamicin pilot study

The results from the rat-gentamicin pilot study showed that the histopathological changes were most pronounced in the gentamicin-ioversol group. *Figures 12 a - f* illustrates the histomorphological findings.



a. Gentamicin-saline *b. Saline-ioversol* *c.Saline-saline*

d. Gentamicin-ioversol *e. Gentamicin-ioversol* *f. Gentamicin-ioversol*

Figure 12 Representative pictures from the rat-gentamicin pilot study (400 times magnification). Kidney tissue was stained with hematoxylin and eosin. a) Gentamicin-saline – note the increased eosinophilia (arrow) within the proximal tubules, due to the hyaline droplets, compared with the saline-saline. b) Saline-optiray – note the small clear vacuoles (V) present in the proximal tubules. c) Saline-saline. d, e, f) Gentamicin-ioversol – note the extensive necrosis of the proximal tubules (N) and the surround interstitial inflammation (arrow).

Histopathological findings in the gentamicin-ioversol group revealed hydropic degeneration and necrosis of the proximal tubular epithelium, protein cast formation, and a chronic interstitial inflammation. These findings were not present in any of the other treatment groups.

Furthermore, several of the biomarkers were elevated in the gentamicin-ioversol group. *Table 9* show that there was a marked rise in NAG, creatinine, and total protein in the gentamicin–ioversol group compared to the other groups.

Table 9 Biomarkers in the pilot study.

	Mean percent change in urinary excretion from baseline to day 4			
Biomarker	Saline- Saline	Saline - Optiray	Gentamicin-Saline	Gentamicin -Optiray
GGT	-83	-41	62	-78
NAG	-45	-237	450	828
Creatinine	-57	-0,9	151	212
Total protein	-41	-99	115	392

GGT= γ-glutamyl transferase, NAG = N-acetyl-β-glucosaminidase.

Because of these results, a more comprehensive study to optimize this model further was done. This was required to optimize the dose of gentamicin that gives the required level of nephrotoxicity, to verify the dose required in younger rats, and to elucidate potential biomarkers for nephrotoxic effects of iodinated x-ray contrast media.

4.2.2 The rat-gentamicin model

Average weight during the study

The weight curve gives an indication of the animals well being. No major decrease in body weights were observed during the study. The animals in the three gentamicin groups and in the gentamicin-ioversol group had a body weight gain similar to the control animals (saline).

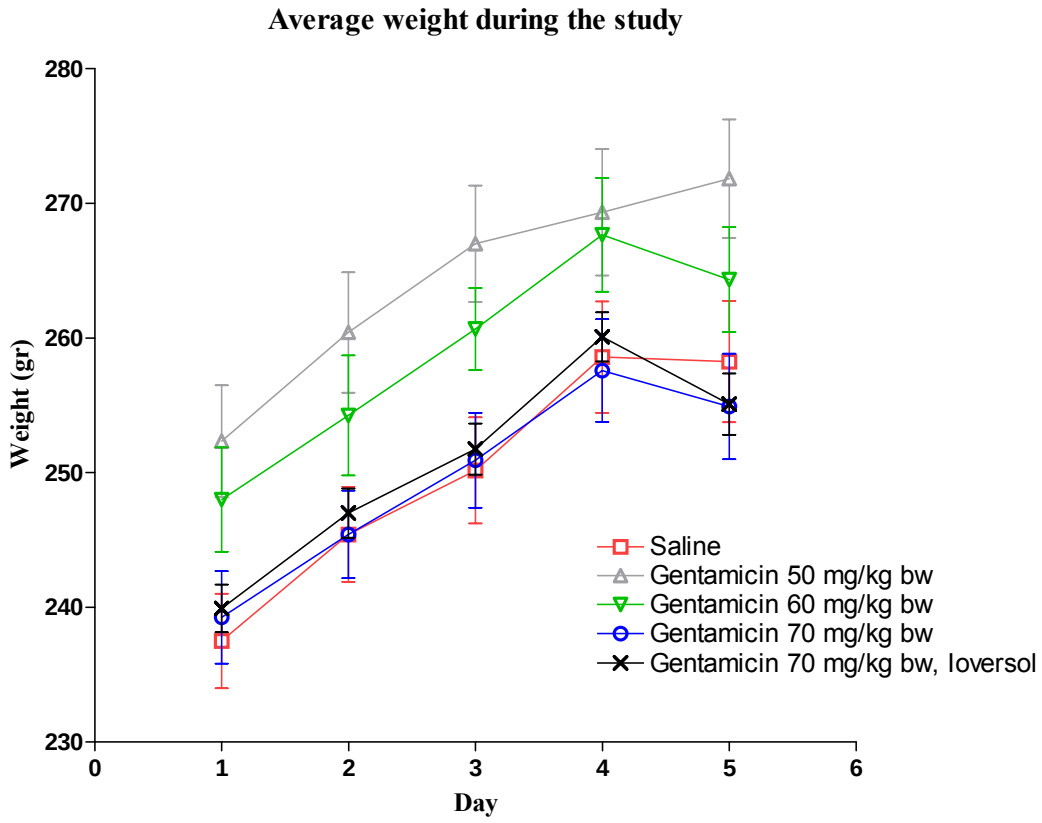


Figure 13 Average weight for the five groups during the study. Mean ± SEM are plotted. n = 12 for all groups.

The reason why the weight tends to decrease on day 5 is that the animals were weighed after blood sampling (approximately 1.3 ml) and euthanization (loss of urine and faeces). When mean weight gain from day 1 to day 4 was analysed by Kruskal-Wallis ANOVA on ranks ($p < 0.05$, $n = 12$), no statistically significant differences were detected between the groups.

Gross pathology

In the control group (saline), all the kidneys looked normal. In the gentamicin 50 mg/kg bw group, some of the kidneys were a little pale compared to the control group. The degree of paleness increased further with the dose of gentamicin administered and was most pronounced in the gentamicin-ioversol group.

The gentamicin-ioversol group was the only group where kidney lesions were detected. This was seen in four of the kidneys (of 24 in total) in this group. One kidney had a small (1 – 3 µm) focal lesion which was yellow and round, two kidneys (same animal) had a small multifocal depression, and the last kidney had a small depression in the form of a cranial pole.

Kidney weight

The kidneys were the only organ taken out and weighed in this study. *Table 10* illustrates the increase in kidney weight (both left and right) given as percentage of total body weight in the treatment groups.

Table 10 Right and left kidney in percentage of total body weight for the five groups in the study

	Right kidney in % of bw Mean ± SEM	Left kidney in % of bw Mean ± SEM
Saline	0.37 ± 0.01	0.37 ± 0.01
Gentamicin 50 mg/kg bw	0.39 ± 0.00 *	0.38 ± 0.01
Gentamicin 60 mg/kg bw	0.39 ± 0.00 *	0.38 ± 0.01
Gentamicin 70 mg/kg bw	0.39 ± 0.01 *	0.39 ± 0.01 *
Gentamicin 70 mg/kg bw and Ioversol	0.44 ± 0.01 *	0.42 ± 0.01 *

** indicates statistically significant difference relative to the control (saline) (one way ANOVA and Dunnet's post hoc test. n = 12. p < 0.05).*

All treatment groups showed to have a statistically significantly higher right kidney weight than the control group. The kidney weight increased with the dose of gentamicin administered and was highest in the gentamicin-ioversol group. Only the gentamicin 70 mg/kg bw and the gentamicin-ioversol group had a statistically significantly higher left kidney weight than the control group. Also for the left kidney, the weight increased with the administered dose of gentamicin and was highest in the gentamicin-ioversol group.

The differences in the groups are minor, but the variances are low and therefore statistically significant differences were found. For both right and left kidney weight, the mean in the

treatment groups are higher than for the saline group, and the kidney weight tends to increase with the administered dose of gentamicin and was most pronounced in the gentamicin-ioversol group.

Histopathology

The histopathological findings are listed in *Table 11*. Gentamicin-related histopathological findings were noted in the kidneys at all doses. Hyaline droplet formation in the proximal tubular epithelium was observed in 10/12 rats at 50 mg/kg bw and 60 mg/kg bw, and 12/12 at 70 mg/kg bw. The severity was dose-related and ranged from minimal to marked. The hyaline droplets were characterized as multiple small dense, rounded or angular intracytoplasmic bright eosinophilic-hyaline-droplets. The effected tubules were present in the diffusely throughout the outer cortex. Interstitial inflammation was also noted in 1/12 animals at 50 mg/kg bw and 2/12 animals at 70 mg/kg bw. The severity ranged from minimal to mild. The change consisted of focal infiltrates of lymphocytes and a smaller number of plasma cells inbetween the proximal tubules.

The gentamicin-ioversol treatment group was associated with histopathologic findings in the kidney that consisted of hyaline droplet formation, tubular vacuolation, tubular dilatation, tubular necrosis, and interstitial inflammation. The hyaline droplet formation had characteristic features identical to those described in the gentamicin-treatment only groups. The severity was minimal to severe and the finding was present in all animals. The proximal tubular vacuolation consisted of numerous small spherical colourless vacuoles contained within and expanding the cytoplasm of proximal tubular epithelium. The vacuolation was present in all segments of the proximal tubule. The vacuolation was noted in all treated animals and the severity was minimal to moderate. In all cases the tubular lesions were present in the proximal tubules and all lesions were noted in the outer cortex of the kidney only. Tubular dilatation was noted focally throughout the cortex. This change consisted of proximal tubule with wide open lumens and occasionally flattened epithelium. The finding was present in 6/12 animals and the severity was minimal to marked. The proximal tubule necrosis noted consisted of focally distributed proximal tubules with denuded or degenerated/necrotic epithelium. Numerous tubules contained amorphous eosinophilic material or cellular debris and in some cases protein casts were noted in the more distal segments of the nephron. The tubular necrosis was noted in 8/12 animals and the severity

ranged from minimal to severe. The interstitial inflammation had the characteristic features identical to those described in the gentamicin-treatment only groups. The inflammation was mutlifocally distributed throughout the affected kidneys and was often co-localised with areas of tubular necrosis. The lesion was noted in 6/12 animals and the severity ranged from minimal to severe. The control (saline) revealed no histopathological abnormalities.

Table 11 Summary of histopathological changes related to gentamicin and gentamicin-ioversol treatment in male Sprague Dawley rats.

Dose - Gentamicin (mg/kg)	0 (Saline)	50	60	70	70
Dose - Ioversol (g I/kg)	0	0	0	0	6
Number of Animals	12	12	12	12	12
Kidney					
Proximal Tubule					
Hyaline droplet formation	0/12	10/12	10/12	12/12	12/12
Not present	12	2	2	-	-
Minimal	-	6	3	1	1
Mild	-	3	4	4	4
Moderate	-	1	3	5	4
Marked	-	-	-	2	2
Severe	-	-	-	-	1
Vacuolation	0/12	0/12	0/12	0/12	12/12
Not present	12	12	12	12	-
Minimal	-	-	-	-	4
Mild	-	-	-	-	7
Moderate	-	-	-	-	1
Marked	-	-	-	-	-
Severe	-	-	-	-	-
Tubular dilatation	0/12	0/12	0/12	0/12	6/12
Not present	12	12	12	12	6
Minimal	-	-	-	-	2
Mild	-	-	-	-	2
Moderate	-	-	-	-	1
Marked	-	-	-	-	1
Severe	-	-	-	-	-
Necrosis	0/12	0/12	0/12	0/12	8/12
Not present	12	12	12	12	4
Minimal	-	-	-	-	2
Mild	-	-	-	-	2
Moderate	-	-	-	-	2
Marked	-	-	-	-	1
Severe	-	-	-	-	1
Interstitium					
Inflammation	0/12	1/12	0/12	2/12	6/12
Not present	12	11	12	10	6
Minimal	-	1	-	1	3
Mild	-	-	-	1	2
Moderate	-	-	-	-	-
Marked	-	-	-	-	-
Severe	-	-	-	-	1

The histopathological changes described in *Table 11* are illustrated in the following pictures (*Figure 14 a - g*).

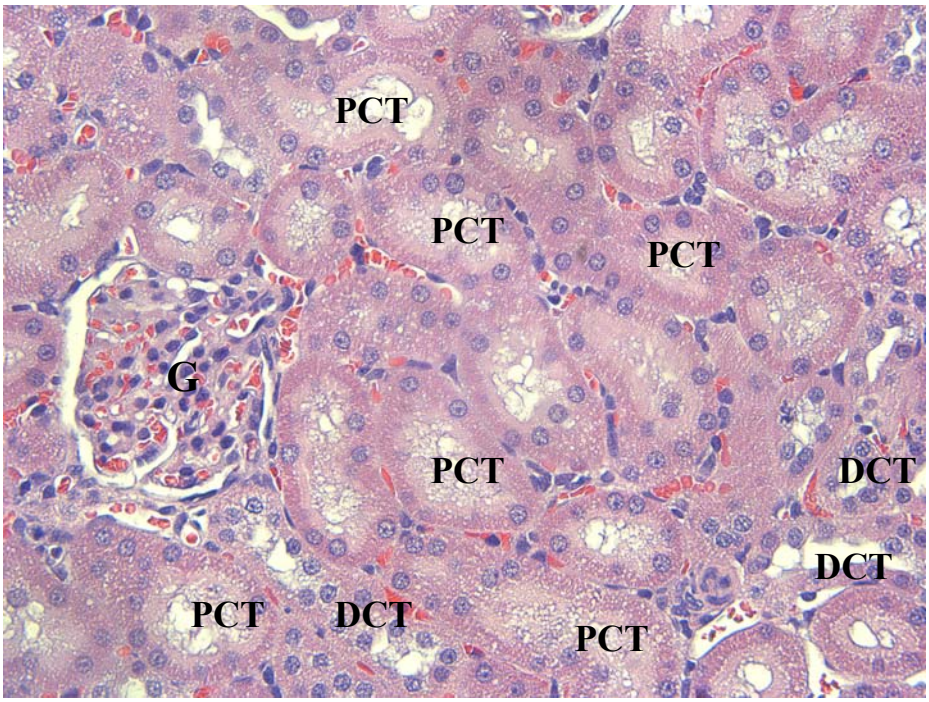


Figure 14 a. Hematoxylin and eosin stained kidney tissue from saline treated animal (control), renal cortex outer stripe (x400). G = glomerulus, PCT = proximal convoluted tubule, DCT= distal convoluted tubule.

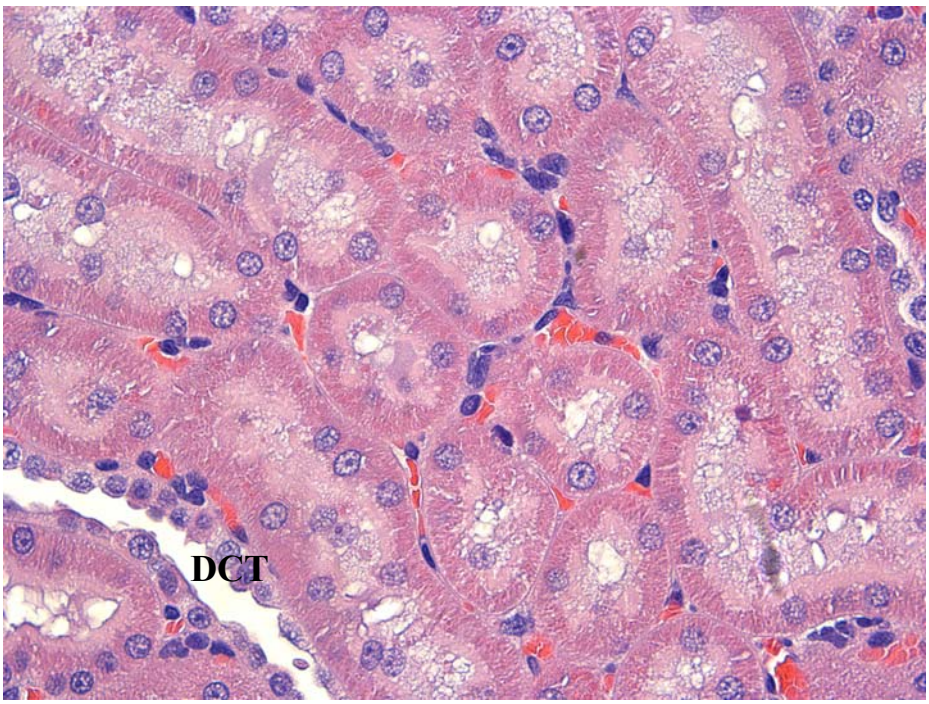


Figure 14 b. Hematoxylin and eosin stained kidney tissue from saline treated animal (control), renal cortex outer stripe (x600). All the tubules present, apart from the one distal convoluted tubule (DCT), are proximal convoluted tubules.

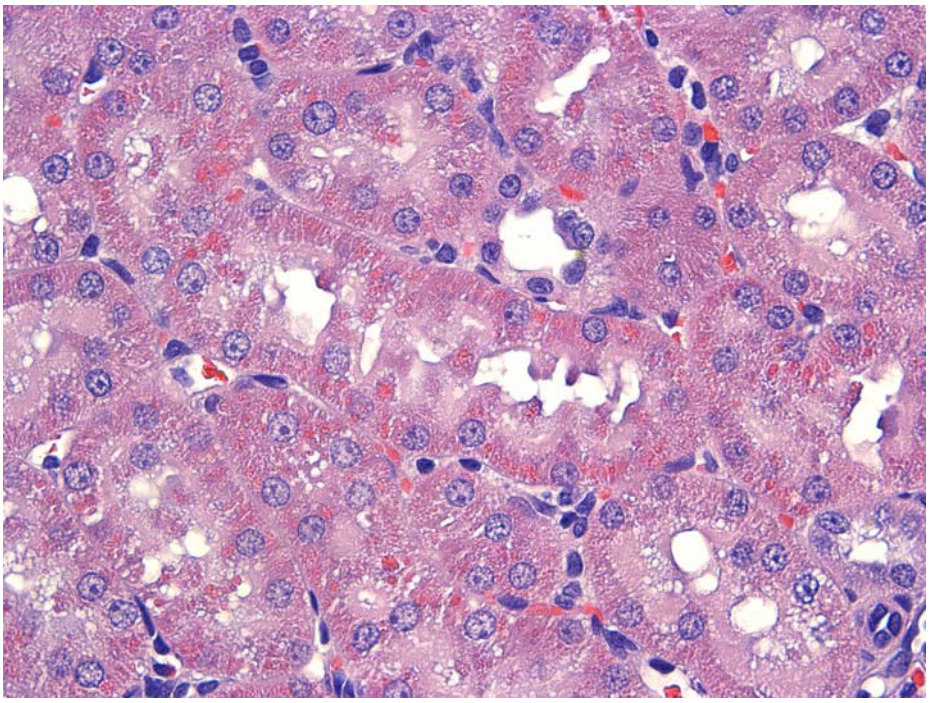


Figure 14 c. Hematoxylin and eosin stained kidney tissue from gentamicin treated animal (60 mg/kg bw) (x600). Note the increase number of small intracytoplasmic eosinophilic droplet in the proximal convoluted tubules (compared to the saline control).

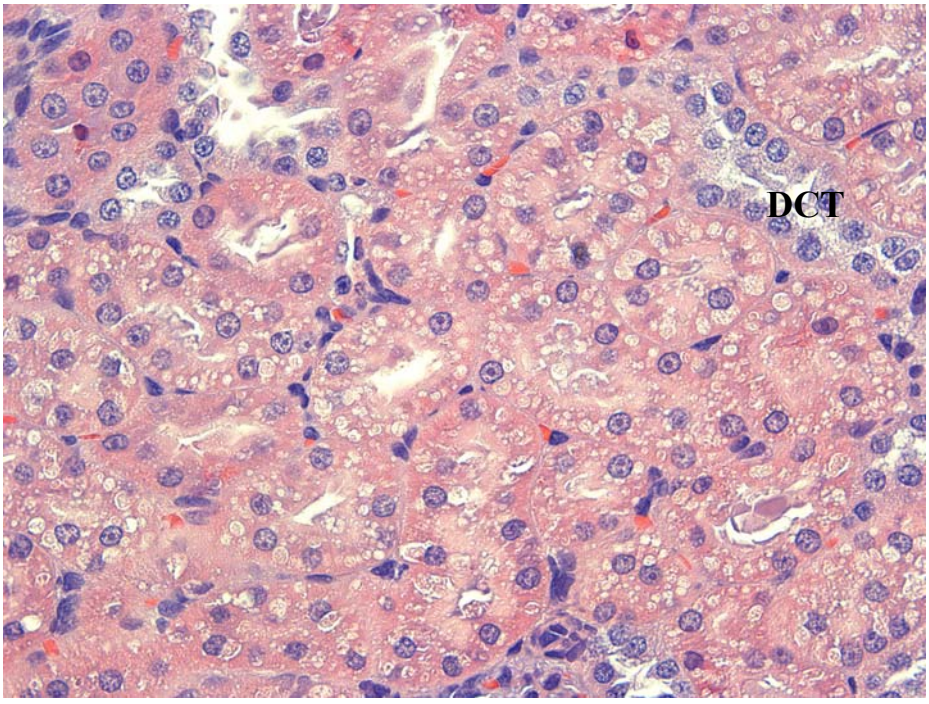


Figure 14 d. Hematoxylin and eosin stained kidney tissue from gentamicin-ioversol treated animal (x600). Note the numerous vacuoles present in the proximal convoluted tubules. The distal convoluted tubules (DCT) are not affected.

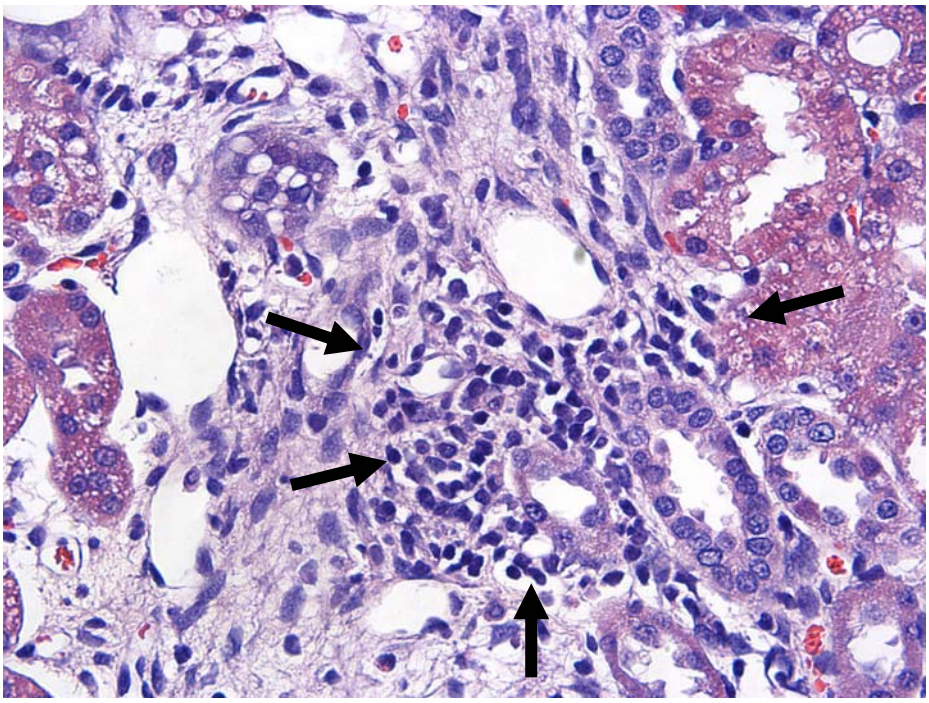


Figure 14 e. Hematoxylin and eosin stained kidney tissue from gentamicin-ioversol treated animal (x600). Note the minimal infiltrate of numerous lymphocytes (cells containing small darkly stained nuclei) that dissect between the tubules.

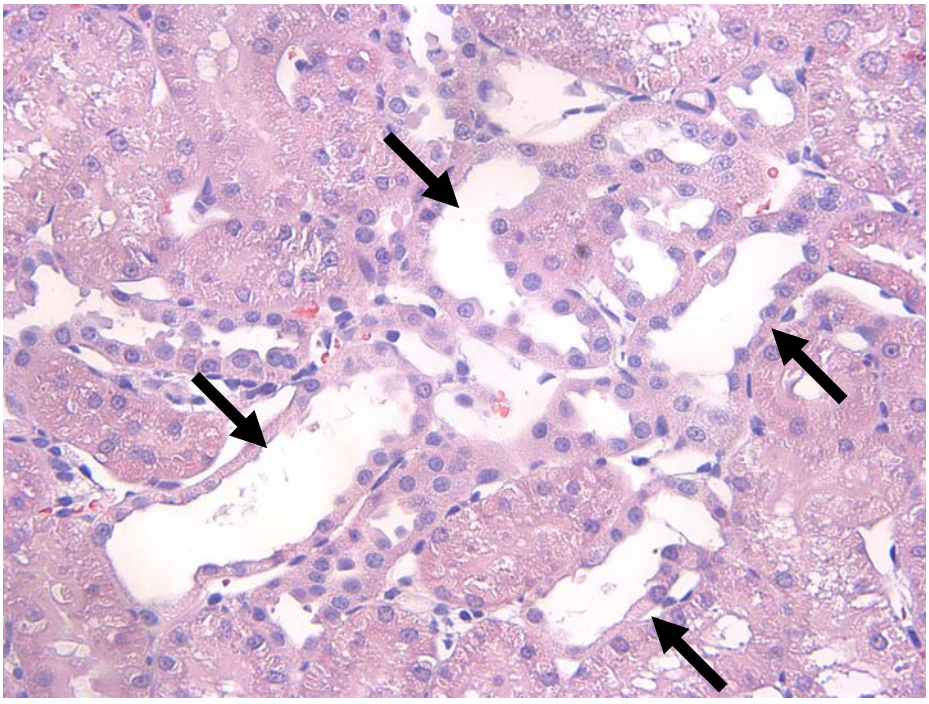


Figure 14 f. Hematoxylin and eosin stained kidney tissue from gentamicin-ioversol treated animal (x400). Note the dilated tubules (arrows). Such tubules were multifocally scattered throughout the kidneys of affected animals.

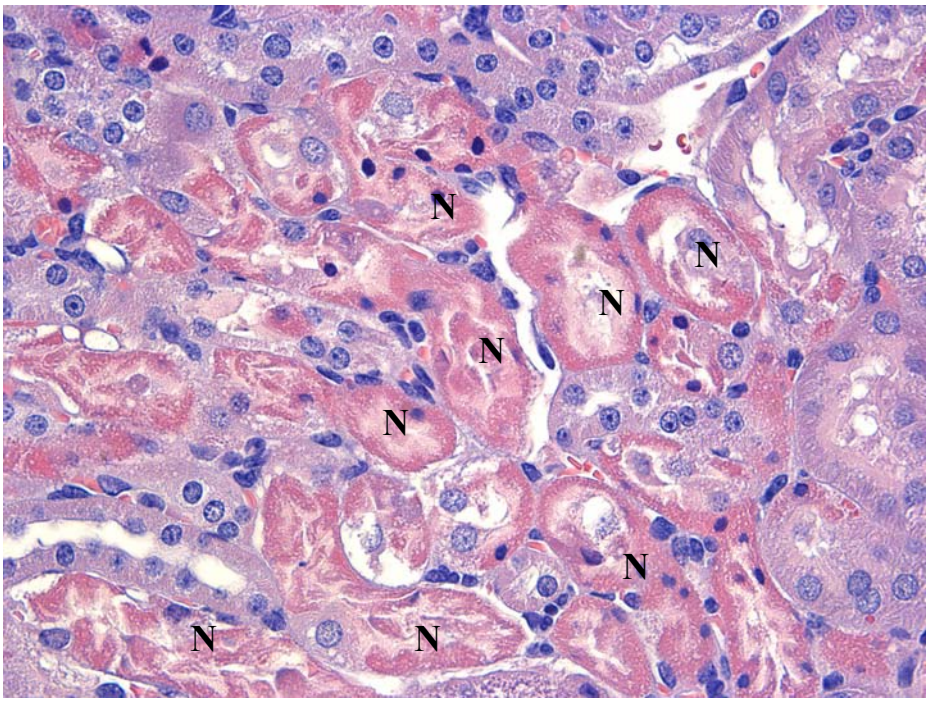


Figure 14 g. Hematoxylin and eosin stained kidney tissue from gentamicin-ioversol treated animal (x600). Note the numerous necrotic tubules (N). The tubules were characterized by indistinguishable cell morphology, pyknotic or fragmented nuclei and an eosinophilic material contained within the tubules.

Serum biomarkers of renal damage

Creatinine and cystatin C was measured in rat serum to detect possible differences before and after treatment.

Creatinine

No statistically significant differences were detected for serum creatinine (µmol/l) from baseline (day -3) to day 5. The standard variations observed in the groups were large, and it was not possible to see a trend in the data.

Table 12 Percent change in serum creatinine for the groups of the study

Serum creatinine (percent change from baseline to day 5)				
	Mean	SD	SEM	p < 0.05 *
Saline	10.02	27.41	7.91	
Gentamicin 50 mg/kg bw	10.48	25.19	7.27	No
Gentamicin 60 mg/kg bw	2.72	17.62	5.09	No
Gentamicin 70 mg/kg bw	17.85	22.07	6.37	No
Gentamicin 70 mg/kg bw and Ioversol	5.95	13.87	4.00	No

** indicates statistically significant difference relative to the control (saline) (one way ANOVA, n = 12, p < 0.05).*

Cystatin C

The cystatin C level was below the detection limit of the ELISA kit (200 ng/ ml) for all serum samples. The samples were analysed at the lowest dilution possible (4 times dilution). Dilutions beyond that were not recommended due to an increased risk of matrix effects.

Urine biomarkers of renal injury

Creatinine, NAG, GGT, total protein, α-GST, and cystatin C were measured as urine parameters to elucidate potential biomarkers of renal injury caused by ICM. Each rat served as its own control, and hence the percent change from baseline (day – 3) to day 4 or day 5 is most widely used to express the results in this chapter.

Creatinine

No statistically significant differences were detected in total urinary creatinine excretion (mmol/l) from baseline to day 4 between the control group (saline) and the four treatment groups.

Table 13 Total urinary excretion of creatinine (mmol/l)

Total urinary excretion of creatinine (percent change from baseline to day 4)				
	Mean	SD	SEM	p < 0.05 *
Saline	33.78	12.62	3.81	
Gentamicin 50 mg/kg bw	28.67	19.78	5.71	No
Gentamicin 60 mg/kg bw	29.90	15.80	4.56	No
Gentamicin 70 mg/kg bw	34.04	15.84	4.78	No
Gentamicin 70 mg/kg bw and Ioversol	19.58	20.64	5.96	No

** indicates significantly difference relative to control (saline) (one way ANOVA and Dunnet's post hoc test, 11 ≤ n ≤ 12, p < 0,05).*

Since no statistically significant differences in urinary creatinine excretion was observed, it should not be a source of error to correlate the other urine parameters to urine creatinine.

Total protein

The results from urinary protein analysis showed that total urinary protein (mg/dl) increased with the administered dose of gentamicin, with the exception of the gentamicin 70 mg/kg bw group, and was most pronounced in the gentamicin-ioversol group.

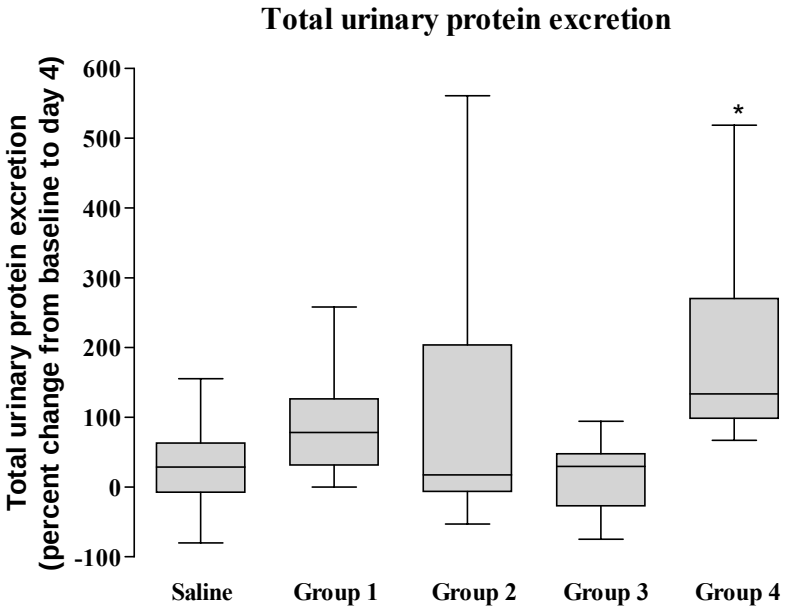


*Figure 15 Median and quartiles for the groups in the study. * indicates significantly difference relative to the control group (Kruskal-Wallis ANOVA on ranks with Dunn's post hoc test. 11 ≤ n ≤ 12, p < 0.05). Saline = control, group 1 = gentamicin 50 mg/kg bw, group 2 = gentamicin 60 mg/kg bw, group 3 = gentamicin 70 mg/kg bw and group 4 = gentamicin 70 mg/kg bw and ioversol.*

The only group that showed to be statistically significantly higher than the control group (saline) was the gentamicin-ioversol group. Even though no statistically significant differences in urinary protein excretion change were detected between the control group and the other treatment groups, quite large differences were observed.

N-acetyl-β-glucosaminidase

The results showed that there were large differences in NAG activity (IU/mmol creatinine) between the different treatment groups and the control group (saline). The percent change in urinary NAG activity increased with the dose of gentamicin administered and was most pronounced in the gentamicin-ioversol group.

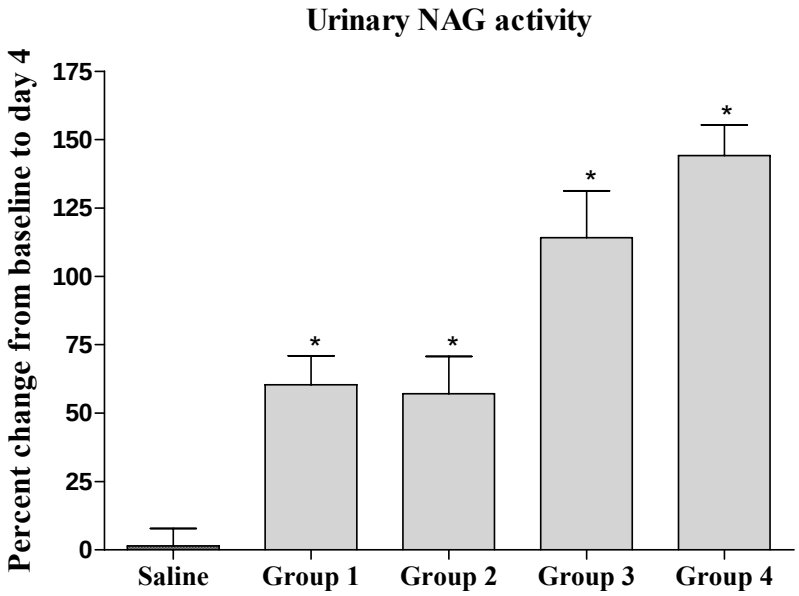


*Figure 16 Percent change in urinary NAG activity (IU/mmol creatinine) from baseline (day – 3) to day 4 for the different treatment groups of the study. Mean ± SEM are plotted. * indicates significant difference relative to control (one way ANOVA with Dunnet's post hoc test, 9 ≤ n ≤ 12, p < 0.05). Saline = control, group 1 = gentamicin 50 mg/kg bw, group 2 = gentamicin 60 mg/kg bw, group 3 = gentamicin 70 mg/kg bw and group 4 = gentamicin 70 mg/kg bw and ioversol.*

The percent change in NAG activity from baseline to day 4 was statistically significantly higher in all treatment groups when compared to the control group. When the change in NAG activity in the treatment groups were further compared pairwise with Duncan's post hoc test, no statistically significant differences were detected between gentamicin 70 mg/kg bw and the gentamicin-ioversol group, but they were both statistically significantly higher than gentamicin 50 mg/kg bw and gentamicin 60 mg/kg bw.

γ-Glutamyl transferase

The percent change in urinary GGT activity (IU/mmol creatinine) from baseline to day 4 increased with the dose of gentamicin administered, with the exception of gentamicin 70 mg/kg bw group, and was most pronounced in the gentamicin-ioversol group.

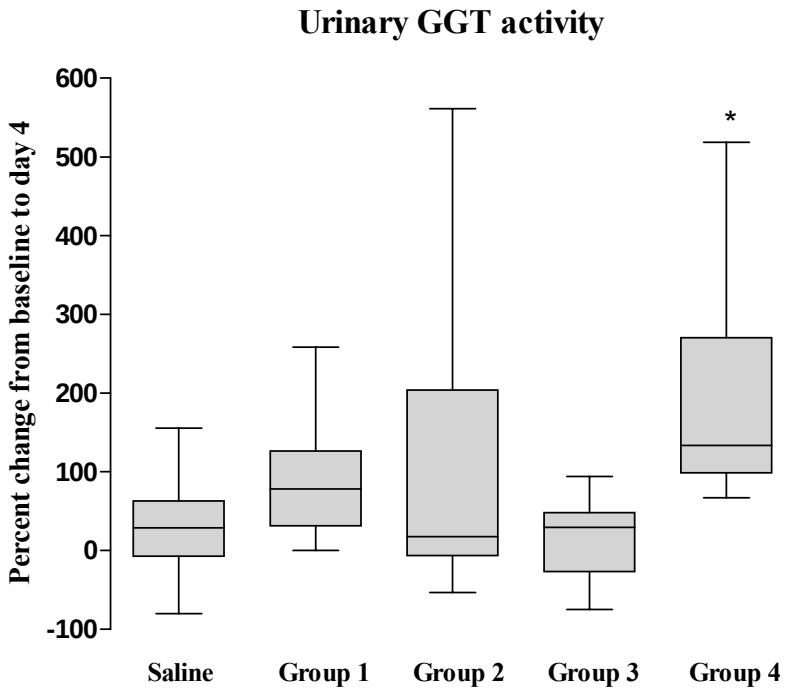


*Figure 17 Percent change in urinary GGT activity (IU/mmol creatinine) from baseline (day –3) to day 4 for the groups in the study. * indicates significant difference relative to the control group (Kruskal-Wallis ANOVA on ranks with Dunn's post hoc test. 11 ≤ n ≤ 12. p < 0.05). Saline = control, group 1 = gentamicin 50 mg/kg bw, group 2 = gentamicin 60 mg/kg bw, group 3 = gentamicin 70 mg/kg bw and group 4 = gentamicin 70 mg/kg bw and ioversol.*

The percent change in urinary GGT activity in the gentamicin-ioversol group was statistically significantly higher than the control group (saline). No statistically significant differences were observed when the other treatment groups were compared to the control group (saline). The urinary GGT activity in the gentamicin-ioversol group was statistically significantly higher than in the gentamicin 70 mg/kg bw group.

α-Glutathione S-transferase

The result from the α-GST analysis showed large mean differences in the percent change in urinary α-GST excretion from baseline to day 4 for most of the treatment groups, however no statistically significant differences were detected when they were compared with the control group. The gentamicin-ioversol group showed the highest increase in urinary α-GST excretion compared to the control group.

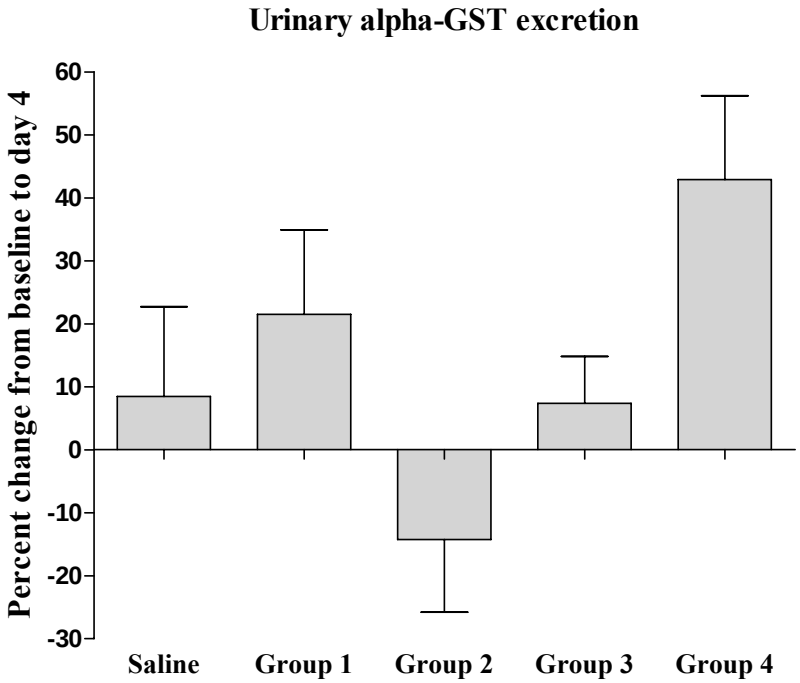


Figure 18 Percent change in urinary excretion of α-GST (µg/mmol creatinine) from baseline to day 4 for the treatment groups of the study. Mean ± SEM are plotted. No statistically differences were detected (one way ANOVA. 10 ≤ n ≤ 12, p < 0.05). Saline = control, group 1 = gentamicin 50 mg/kg bw, group 2 = gentamicin 60 mg/kg bw, group 3 = gentamicin 70 mg/kg bw and group 4 = gentamicin 70 mg/kg bw and ioversol.

Cystatin C

All urine samples were below detection limit of the ELISA (200 ng/ml). The samples were analysed at the lowest dilution possible (20 times dilution). Dilutions beyond that were not recommended due to the increased risk of matrix effects.

Urine cytology

Quantitative results

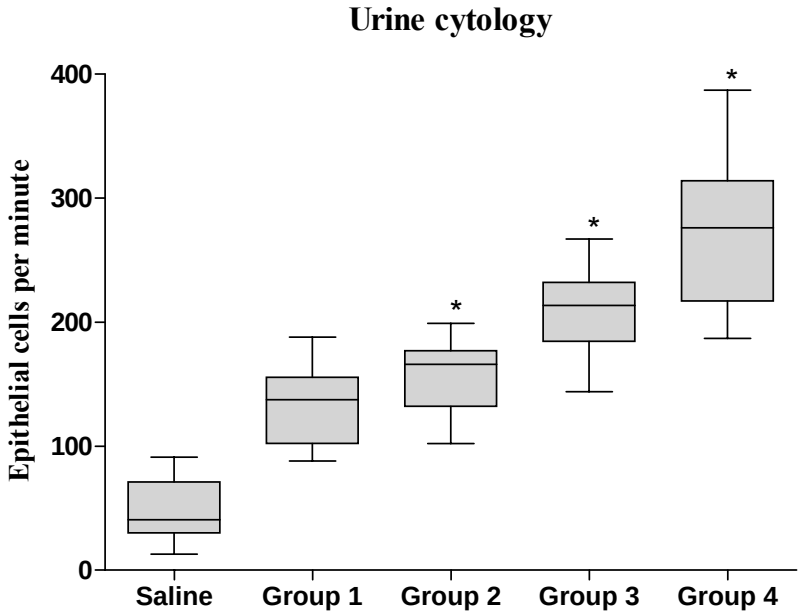


*Figure 19 Epithelial cells/min assessed on urine sediment preparations collected on day 4. * indicates significant difference relative to the control group (Kruskal-Wallis ANOVA on ranks with Dunn's post hoc test. 11 ≤ n ≤ 12. p < 0.05). Saline = control, group 1 = gentamicin 50 mg/kg bw, group 2 = gentamicin 60 mg/kg bw, group 3 = gentamicin 70 mg/kg bw and group 4 = gentamicin 70 mg/kg bw and ioversol).*

The gentamicin 60 mg/kg bw, gentamicin 70 mg/kg bw, and the gentamicin-ioversol group had a statistically significantly higher release of epithelial cells per minute than the control group.

When the groups were compared pairwise (Kruskal-Wallis ANOVA on ranks with Dunn's post hoc test, $p < 0.05$) the gentamicin-ioversol group showed to release statistically significantly more epithelial cells per minute than the gentamicin 50 mg/kg bw and the gentamicin 60 mg/kg bw group. No statistically significant difference was detected between the gentamicin 70 mg/kg bw and the gentamicin-ioversol group.

Morphological analysis results

The Papanicolaou stain cytospin preparations revealed a dose-related increase in the number of viable and necrotic renal tubule cells in the gentamicin-treatment groups. The predominant cell type was the convoluted renal tubule cell indicating the proximal tubules as the site of origin. The convoluted renal tubule cells were large (40 – 75 μm in diameter) and contained

abundant, coarsely granular eosinophilic cytoplasm, indistinct cell borders, and a round to oval, often pyknotic nuclei position at one pole of the cell.

The gentamicin-ioversol treatment group had a higher number of total renal tubule cells per 10 HPF and this group contained a higher proportion of necrotic renal tubule cells when compared with the 70 mg/kg bw gentamicin only group. Furthermore numerous renal epithelial fragments were noted in the gentamicin-ioversol treatment group.

5. Discussion

5.1 Statistical analysis

There are three main factors affecting the outcome of statistical analysis; (1) the number of observations or replicates of an experiment; (2) the distribution in the data; (3) the difference in means. Skewness or abnormalities in any of these three points might make the interpretation of the results more complicated than they really are. In this master's thesis, most of the results are clear and in many cases the differences between the groups can be seen without statistical analysis.

In the results from the trypan blue exclusion test, the criteria for normal distribution and homogeneity of variance were fulfilled in the majority of the experiments. However, at some time points (12 and 24 hours) these criteria were not fulfilled. Since the majority of the experiment fulfilled the criteria, normal distribution and homogeneity of variance was assumed for the total data set.

After 12 hours exposure, it was the control group which did not pass the normality test and the homogeneity of variance test. The 20 experimental values in that group varied from 4 % to 8 %, and due to overall little variance (9 replicates with 6 % cell death), the group did not pass the tests. In the case of percent cell death in a control group, a variation between 4 % and 8 % should be considered as a normal biological variation. Therefore, the data set at 12 hours exposure was assumed to be of normal distribution and with equal variance.

After 24 hours exposure, the control did not pass the normality test and the homogeneity of variance test. At this time point a higher variation in the experimental values were observed, ranging from 4 % to 14 % (6 replicates of 6 % and 5 replicates of 7 %, strong right skewness in the data). However, from a biological point of view, this should be considered to be normal. The ioversol group at 24 hours passed the equal variance test, but not the normality test. One experimental value in the ioversol group (40 % cell death) was the reason why the group did not pass that test.

Taken together, the reason why the experimental values did not pass the normality

distribution test and the equal variance test was due to “low” variance at 12 hours, a tail skewed to the right in the control group at 24 hours, and because of one experimental value within the ioversol group at 24 hours exposure. This clearly indicates that the majority of the experimental values are within the criteria for normal distribution and equal variance, and therefore it seems fair to assume normal distribution and equal variance in the total data set and perform parametric statistical tests over non-parametric. For the benefit of the reader, the results from these time points are presented with non-parametric analysis in appendix 2.

5.2 *In vitro* and *in vivo* models versus human data

Whether the toxicity observed and reported in *in vitro* and animal studies are applicable to human patients is questionable. However, the currently preferred way to improve our understanding of problems related to administration of ICM is through *in vitro* and *in vivo* experiments.

A good way to elucidate underlying mechanisms of CIN and to study the direct toxic effect of ICM in the absence of confounding variables is through *in vitro* experiments. Therefore, *in vitro* studies are considered to be essential research tools for such investigations.
Both primary cells and cell lines have previously been used to evaluate toxic effects of ICM. In primary cells, most of the differentiated epithelial functions are maintained, and hence these cells are the most relevant for toxicological studies (Idee and Bonnemain, 1996). However, primary cells have a limited life-span, and it takes expertise to isolate and cultivate them and that limits their use. Alternatively, several cell lines of renal origin are available from a variety of species, but these cells have lost much of their differentiation and are therefore of less clinical relevance than primary cells. However, cell lines have several advantages; they are easy to obtain, maintain their characteristics for extended periods, can be replaced easily, have well described optimal culture conditions, and are well described. Cell lines are therefore more frequently used than primary cultures. Cells grown in optimal *in vitro* conditions are healthy and viable, and high doses of ICM and long exposure periods are required to induce cell death. A few studies have tried to sensitize the cells before exposure to ICM to resemble the true situation. Cunha and Schor (2002) exposed LLC-PK1 cells to gentamicin alone and together with lipopolysaccarides for 48 hours before exposure to ICM. Wasaki et al. (2001) exposed primary mesangial cells to 3 different concentrations of sucrose

before exposure to ICM to mimic diabetic patients. Both studies succeeded in making the cells more susceptible than normal cells, but whether these two "models" are more representative than normal cells remain unknown.

In the case of animal studies, it is normal to induce some kind of renal insult before administration of ICM to increase the sensibility of the kidneys to ICM, and hence these models are considered more similar to the clinical situation. Unfortunately, it is not easy to create a model that fulfils all the criteria for a good model of CIN (see section 1.6.1). Over the previous decades of this area of research, many models have been reported for CIN which are not likely to represent the clinical picture. Dehydration models and administration of ICM in normal animals are such examples. In dehydration models, dehydrated animals with normal kidney function are injected with large doses of ICM. Contrary, patients at risk of CIN have impaired renal function, are well hydrated, and are injected with normal doses of ICM. The pre-condition before administration of ICM are not the same, and therefore it is questionable whether the observed toxicity in the two situations is comparable or not. In experiments using normal animals, a large dose of ICM are needed to induce renal damage. In the latter example, the observed toxicity might be a result of intoxication, and hence do not represent the same toxicity as observed in CIN in humans. Despite many years of research in this area, there is still no universally accepted animal model of CIN. Thus, a need for a good model for investigation of CIN is still present.

It is important to keep in mind that *in vitro* and *in vivo* models should not be compared directly as they do not provide the same information. They are intended to improve our understanding of different aspects of the same problem.

5.3 Chose of *in vitro* exposure conditions

For all methods used in this thesis, with MTT assay as a possible exception, long exposure periods are required to induce toxicity in NRK 52-E cells. That is in consistence with what is normally observed in *in vitro* studies (Haller and Hizoh, 2004). The long exposure time was selected because it takes time to induce toxicity in healthy and viable cells. Furthermore, it is not irrelevant to the clinical picture as renal retention of low doses of ICM in the medulla has been reported up to 32 hours after administration of ICM (Rickards et al., 1997).

The concentration of the ICM was selected based on previously reported *in vitro* studies (Heinrich et al., 2005;Wasaki et al., 2001), and also based on the fact that ICM are concentrated in the kidneys. Urinary concentrations of ICM can be up to 50 to 100 times that in plasma (Katzberg, 1997). Furthermore, Spataro et al (1982) reported urinary concentration up to approximately 140 mg I/ml at 60 minutes after administration of 450 mg I/kg ioxaglate in rabbits.

5.4 The role of physicochemical properties in *in vitro* iodinated x-ray contrast media toxicity

LOCM has been showed to be better tolerated than HOCM in patients at risk (Rudnick et al., 1995). Furthermore, IOCM has shown to be superior to LOCM in some epidemiological trials (Aspelin et al., 2003). These studies strongly indicate that the osmolality of the contrast agent contribute to the observed toxicity in patients. Moreover, *in vitro* studies on cultured renal cells have also demonstrated that LOCM are less toxic than HOCM (Andersen et al., 1994;Duan et al., 2006;Haller et al., 1997;Schick and Haller, 1999;Schick et al., 2002). Only a few studies have evaluated the toxic effect of IOCM on cultured renal cells (Hardiek et al., 2001;Heinrich et al., 2005;Itoh et al., 2006;Schick and Haller, 1999), and none of these have directly concluded that IOCM are less toxic than LOCM on cultured renal cells. However, the set up and interpretation of the results can be discussed in some of these studies (see section 5.4.4 for details).

Several *in vitro* studies have shown that the toxicity observed in cultured renal cells can not solely be explained by the osmolality of the contrast agent, as mannitol of equal osmolality induces a less pronounced toxicity (Haller et al., 1997;Hardiek et al., 2001;Schick et al., 2002;Zager et al., 2003). This finding suggests that the direct compound toxicity appears to play a dominant pathological role. However, as these studies have not attributed the toxicity to any particular property of ICM, the property that causes toxicity in addition to osmolality remains unknown. The release of inorganic iodine with subsequent iodine toxicity seems not to be involved in the direct toxicity (Zager et al., 2003).

The viscosity of the contrast solution is likely to be more important in enclosed systems, like the kidney, than in an open *in vitro* system. Some recent reviews have expressed their concern regarding the increased viscosity of IOCM and claimed that based on the viscosity, IOCM are no better tolerated than LOCM (Persson and Tepel, 2006;Persson et al., 2005). However, these statements are mainly based on theoretical estimations, and currently there is no evidence supporting that the viscosity of IOCM is of importance to the observed pathophysiology. Recently, a study using a porcine model showed that the viscosity of iodixanol did not have any nephrotoxic effect on renal function (Elmstahl et al., 2006). Overall, the suggestion that the viscosity of IOCM are of importance for the renal pathophysiology remains highly speculative.

The role of additives in the contrast solution (e.g. EDTA) are probably more important in *in vitro* systems at long exposure times than in patients undergoing radiographic examination. Of the four contrast agents evaluated in this present work iodixanol, iohexol, and iopromide all contain 0.1 mg EDTA/ml, whereas ioversol contains 0.2 mg EDTA/ml.

The osmolality measured for all ICM at a concentration of 150 mg I/ml, diluted in incubation medium, is given in the introduction. Based on the osmolality, iodixanol (350 mOsm/kg) was expected to induce the least pronounced toxicity, followed by iopromide (530 mOsm/kg), then ioversol (531 mOsm/kg), and with Iohexol (559 mOsm/kg) expected to induce the most pronounced toxicity.

5.4.1 Cell death assessed by the trypan blue excusion assay

When cell death was assessed by the trypan blue exclusion assay after exposure to ICM over different time periods, statistically significant changes were detected already at early time points. However, caution should be taken not to over-interpret these findings. Cell death up to 14 % was detected in the controls, and therefore cell death at approximately this value should be considered normal. The trypsinisation and centrifugation step in the trypan blue exclusion assay might be a source of cell death on its own, and hence some variability in cell death should be expected.

At early exposure time points (up to 6 hours) the cell death reported for iodixanol, iohexol, and iopromide are generally lower than what is reported for the control group. Berg et al.

(2005) showed that iodixanol, iohexol, ioxaglate, and diatrizoate (contrast agents from all classes of ICM) all possess antioxidant properties *in vitro*. It is tempting to speculate that antioxidant properties of the ICM contribute to the lower cell death reported at early time points, but further and more comprehensive experiments are needed to confirm this suggestion.

At 12 and 24 hour exposure, the cell death can be explained by the osmolality of the ICM, as the cell death increases with increasing osmolality. At both time points, iohexol caused the most pronounced cell death, iopromide and ioversol caused a relatively similar and intermediate cell death, whereas iodixanol caused the least pronounced cell death. Statistically significant differences were shown; iohexol caused a statistically significantly higher cell death than iopromide and ioversol, which again caused a statistically significantly higher cell death than iodixanol. Furthermore, the regression analysis that was applied on cell death plotted against osmolality showed a relatively good correlation between them (r^2 = 0.76).

When cell death was assessed by the trypan blue exclusion test, the three non-ionic monomers showed a marked difference in their potential to induce cell death, with iohexol > ioversol > iopromide induced cell death. This finding suggests that the chemical structure of the contrast agents may be related to some of the observed toxicity. However, the three agents have a very similar chemical structure and it is not known which, if any, chemical group on the contrast agents that could cause the toxicity.

5.4.2 Cell viability assessed by the MTT assay

Already at early exposure times the cell viability (as determined by MTT reduction) is dramatically decreased. Hence, it is obvious that the MTT assay is too sensitive to detect early reductions in cell viability caused by ICM.

Statistically significant differences between the four ICM and their potential to reduce MTT conversion were detected at early time points (at 1, 2 and 3 hour exposure). However, after 0.5 hour and 6 hours exposure, no statistically significant differences were detected between the four ICM and their potential to inhibit mitochondrial dehydrogenase activity. The reduction in MTT conversion between 1 and 6 hours exposure were highly variable and went up and down. Hence, these statistically significant differences at early time points are

considered not to have any toxicological relevance.

At 24 hour exposure, the difference in potential to reduce MTT conversion are more pronounced than at earlier time point, and iohexol > ioversol > iopromide > iodixanol reduced the MTT conversion. The reduction in MTT conversion caused by the four ICM showed to be statistically significant different from each other when compared pairwise. However, the inhibition of mitochondrial dehydrogenases caused by iohexol, iopromide, and ioversol (6 %, 11 % and 8 % of the control, respectively) after 24 hours exposure did not differ significantly in percent. However, these small differences are considered not to have any toxicological relevance. From a biological point of view, such small differences have no toxicological relevance, as they do all inhibit the mitochondrial dehydrogensaes greatly.

When a regression analysis was applied on osmolality versus reduced cell viability, a strong correlation between them was found ($r^2 = 0.97$).

Overall, the findings from the MTT assay indicate that the observed toxicity is related to the osmolality of the contrast agent as the MTT conversion is reduced with increasing osmolality of the contrast agent, and because of the strong relationship between reduced cell viability and osmolality.

5.4.3 Cell death assessment on formalin fixed NRK 52-E cells

Due to a small number of replicates, no statistical analyses were included when cell death was assessed on formalin fixed NRK 52-E cells. However, the five countings (from two independent experiments) had a small variance and hence, the results can be considered reliable. Iodixanol induce the least pronounced cell death at most time points (≥ 3 hours exposure time), while iohexol, iopromide, and ioversol all caused a higher cell death than iodixanol. Furthermore, the results showed that iohexol, iopromide and ioversol seem to induce a similar cell death at most exposure times. When a regression analysis was applied on cell death versus osmolality a strong correlation between them was found ($r^2 = 0.99$).

Overall, these findings indicate that the induced cell death can be explained by the osmolality of the contrast agent as cell death increases with osmolality.

5.4.4 Conclusion of *in vitro* sudies and the role of physicochemical properties

The osmolality of the ICM seem to be the major factor contributing to the observed cytotoxicity as iohexol > ioversol > iopromide > iodixanol induced cell death or reduced cell

viability in all *in vitro* experiments throughout this present work. Overall, the observed cell death or reduced cell viability is directly correlated to the osmolality of the ICM. Furthermore, when cell death or reduced cell viability was plotted against osmolality and linear regression analyses were applied, a strong correlation was observed. The correlation between cell death (trypan blue exclusion assay) and osmolality was relatively good, whereas the correlation between reduced cell viability (MTT assay) and osmolality, and between cell death (morphological assessment) and osmolality was very strong. The dilution medium (incubation medium) seems to contribute to the osmolality as iodixanol has a higher osmolality at 150 mg I/ml than undiluted. It can be noted that the contributing effect of dilution medium makes the difference in osmolality between iopromide and ioversol smaller than what is the case when they are undiluted.

The difference in cell death caused by the three LOCM was most pronounced in the trypan blue exclusion assay. This finding suggests that the chemical structure may be important for the observed toxicity. In the MTT assay and cell death assessed on formalin fixed NRK 52-E cells the differences between the three LOCM were less pronounced.

No osmolality controls, or any additives, were included in this present work. Therefore, no conclusions can be made about the contribution to the cell death from these. However, as mentioned previously, it has been demonstrated that solutions of equal osmolality induces a less pronounced toxicity than the ICM. Therefore, caution should be taken not to solely explain the cytotoxicity observed in this thesis as a consequence of osmolality.

In all *in vitro* experiments assessing cell death or cell viability, the toxicity induced by iodixanol was statistically significant lower than the ones caused by iopromide, ioversol, and iohexol. To my knowledge, no other *in vitro* studies on cultured renal cells have reported so pronounced difference in cell death based on the osmolality of the ICM.

Heinrich et al. (2005) compared different contrast agents with the MTT assay, and when ICM were compared at equal iodine concentrations (75 mg I/ml), they found that dimeric contrast agents showed a slightly weaker effect on inhibition of mitochondrial dehydrogensases (40 ± 3 % for iodixanol and 41 ± 2 % for iotrolan) than monomers (32 ± 2 % for ioversol and 34 ± 2 % for iomeprol), but this difference was not statistically significant. The finding in this

present work is in conformity with the findings by Heinrich et al. (2005), although much more pronounced differences were observed in this work. Heinrich et al. (2005) also compared the contrast agents at molar basis and concluded that dimeric ICM was statistically significant more cytotoxic than monomers on cultured renal cells. However, this finding is of limited importance, as ICM are dosed in terms of iodine concentration, not molar basis. Dimeric contrast agents contain twice the number of iodine atoms per molecule and are therefore administered at half the molar dose. Therefore, the latter finding is of limited value. This article has been criticised on their study design and interpretation of the results (Skotland and Grant, 2006).

In a study by Itoh et al. (2006) it was shown that iotrolan reduced the cell viability (determined by WST-8 assay; relatively similar to the MTT assay) while iodixanol did not. The two contrast agents have about the same osmolality and hence, other properties than the osmolality must be responsible for the decrease in cell viability induced by iotrolan. This is mainly in accordance with our finding.

Furthermore, two *in vitro* studies on cultured renal cells have included iodixanol in their studies, but they did not reveal any statistically significant differences between iodixanol and LOCM (Hardiek et al., 2001;Schick and Haller, 1999). Hardiek et al. (2001) reported that when five different ICM were compared, no specific property could be attributed to the observed toxicity. The ionicity seemed to have the greatest influence on MTT reduction, followed by the structure of the contrast agent. The ICM which revealed the least pronounced toxicity in the study was iomeprol. Iomeprol is currently the only ICM that does not contain EDTA as an additive in the contrast solution. It can be speculated that in an *in vitro* system like the one reported, with long exposure times (24 hours), the absence of the chelating agent EDTA may account for the reduced toxicity.

In the study by Schick and Haller (1999) iodixanol and iohexol induced the least pronounced toxicity of the four ICM compared. However, the exposure time was only 6 hours. A short exposure time like that might be more clinically relevant, but as shown in the present work, 6 hours exposure is too short of an exposure time to reveal the cytotoxic differences between the contrast agents.

5.5 Comparison of *in vitro* methods

Staining cells with vital dyes, including trypan blue, is a commonly used approach to assess cell death. The advantages of such an approach are its ease of use and low cost. Comparison can be made between different experimental treatments, doses, or exposure times (Loo and Rillema, 1998). However, disadvantages of the method also exist. The method is based on the ability of living cells with an intact cell membrane to exclude the dye. When a cell undergoes apoptosis it retains its cell membrane integrity until late in the apoptotic programme, and the cell will therefore be able to exclude the dye. This leads to an underestimation of the true cell death (Loo and Rillema, 1998). An underestimation of cell death will also occur when cells undergoes necrotic cell death. The cell membrane of a cell undergoing necrosis will rupture and all the cellular content will be released to the area around the cell (Harriman and Schnellmann, 2005). When rupture of the cell membrane occurs, it is no longer possible to count the cell. In experiments using the trypan blue assay, a blue smear was sometimes seen when the cell preparation was counted. A smear of that type is most likely a smear of cell fragments, fragile dead, or dying cells. Therefore, the true cell death was probably underestimated.

Special caution was taken to include possible dead and detached cells after exposure. To perform the trypan blue exclusion test as such (including collection of cell media, trypsinisation, and centrifugation) it is time consuming compared to other alternatives (e.g. direct counting in the well). However, this way of preparing the cell is the most accurate, as all cells are included. Many studies report on cell death assessed by the trypan blue exclusion assay after exposure to ICM, but not all have taken special caution to include all cells. Andersen et al. (1994) prepared the exposed cells as presented in this thesis, Haller et al. (1997) and Schick et al. (2002) included trypsinisation but did not mention anything about possibly dead and detached cells, while Schick and Haller (1999) discard the medium probably containing dead and detached cells. When the cell medium is discarded, especially after long exposure periods, the true cell death is most likely not reported and differences between ICM are therefore more difficult to detect.

The main advantage of using colormetric assay, like the MTT assay, over dye assays like the trypan blue exclusion assay, is the speed at which the samples can be assayed. Furthermore,

the MTT assay requires no removal or washing steps, which helps to minimize the variability between samples (Mosmann, 1983). However, since there are no washing steps in the assay, ICM are not discarded before the incubation period with MTT. This means that the cells are exposed to the test compound during the 4 hour incubation period with MTT. The exposure is not stopped until MTT solubilization solution is added at the termination of the experiment. Therefore, what is presented as half an hour incubation period is really four and a half hour incubation period. If a comparison were to be made between the MTT and the trypan blue exclusion assay, a 2 hour exposure time in the MTT assay would be the same as 6 hours exposure time in trypan blue exclusion assay. This extra incubation time is a step which most authors disregard when presenting their results (Duan et al., 2006;Heinrich et al., 2007;Heinrich et al., 2005).

Some authors have modified the MTT assay to avoid this problem. They choose to discard the medium before the incubation with MTT (Hardiek et al., 2001). But by doing so, they might include another source of error as it is not healthy for the cells to be incubated for 4 hours without any cell medium. This incubation with MTT solution, without any nutrition, on already impaired cells, might cause cell death on its own. As a consequence of that, the reduced cell viability might be underestimated. Furthermore, when such a modification step is included, more washing and addition of new fluids to the cells are necessary. Special caution has to be taken when fluid is added to the cells. In this present thesis it was observed that when pipetting at a “normal” speed a hole in the confluent cell layer was induced and hence, less MTT was converted to its coloured product. By adding additional washing and adding of fluid steps, the variability of the results may be increased.

Several authors have a tendency to refer to and look upon the MTT assay as solely a cell viability assay. However, this is a wrong and misleading interpretation of the assay, as studies have shown that a decrease in cell viability measured by the MTT assay can be reversed when removing the agent tested and supplying the cells with fresh medium (Hardiek et al., 2001). Hardiek er al (2001) suggested that MTT assay reflects mitochondrial enzyme function rather than cell proliferation or viability. The recovery in mitochondrial dehydrogenase activity after exposure to ICM was also tested in this present thesis, and the results (not shown) were in accordance with Hardiek et al.(2001). The MTT assay should therefore be regarded as a cell viability assay with limitations, and the assay should be used together with other cell viability

or cell death assays. Furthermore, recovery after exposure should be tested, or long exposure times should be included to avoid measuring reversible effects.

In cell death assessment on formalin fixed NRK 52-E cells, cells that were in the process of dying were included as dead cells, and therefore the test is more sensitive than the trypan blue exclusion assay. However, in both the trypan blue exclusion assay and the MTT assay, a special precaution was introduced to include all dead cells (trypan blue exclusion assay) and to not wash away living cells (MTT assay). In the case of cell death assessment on formalin fixed NRK 52-E cells, no such precautions were included. The cover slips containing cells were washed in PBS before formalin fixation, than stained with hematoxylin, and further washed in saline. Both these washing steps might have washed away both dead and living cells and hence a source of error was possibly introduced. However, several washing steps are required to make a preparation of good quality which do not contain wastes and debris, which limits the correct assessment of living, dead, and dying cells.

5.6 Internalization of contrast media *in vitro*

Dobora et al. (1995) suggest that due to higher viscosity of IOCM, the filtrate might have a longer transit time through the kidneys and hence provide an increased opportunity for ICM uptake in the epithelial cells of the lumen. Obviously, this will not be the case with *in vitro* experiments where there are no enclosed spaces where concentration and slower transit time occurs. Therefore, the *in vitro* and *in vivo* situation is not comparable. Nordby et al. (1989) proposed two mechanisms to explain the internalization in cells: (1) ICM may cause a transitory, low grade membrane damage with leakage of extracellular medium into the cells; (2) ICM may pass through leaky channels or be actively transported in by endocytosis or pinocytosis. The first explanation is speculative since membrane damage is not needed in living cells for uptake of ICM. Furthermore, iodixanol has also been used as a marker of fluid phase endocytosis (Kjeken et al., 2001).

To my knowledge there are currently no other reports describing internalization of ICM in cultured cells by using ICP-AES analysis. The results showed an overall high variation, but it was possible to see a trend in the data set. At the 24 hour exposure time point, iohexol > ioversol > iopromide > iodixanol was taken up in the exposed cells. This finding indicates

that uptake of ICM in NRK 52-E cells may be related to the osmolality, as the internalization increases with increasing osmolality. Furthermore, the internalization of ICM was also correlated to the observed cell death or loss of cell viability observed in the *in vitro* experiments, suggesting that cellular uptake of ICM is needed to induce cell death.
In vitro and *in vivo* experiments have shown that IOCM and LOCM are more extensively taken up in cells than HOCM (Dobora et al., 1995; Andersen et al., 1994). Therefore, the internalization of ICM measured by ICP-AES analysis is not in accordance with previous reports. A possible source of error could be that the ICP-AES analysis does not distinguish between ICM that are taken up in the cell and ICM that may be bound to the cell surface. Due the latter fact and the overall high variation in the results, the findings in the ICP-AES experiment should be treated with due caution.

The morphological evaluation preformed on the NRK 52-E cells after exposure to the four ICM showed considerable vacuolisation after 6 hours exposure. The observed vacuolization was most pronounced in the iodixanol treated cells, where numerous vacuoles were observed in the cytoplasm. This was also partly observed in the iohexol, iopromide and ioversol treated cells, where numerous small clear round vacuoles were observed. The morphological observations indicated that iodixanol > iohexol > iopromide > ioversol was taken up in the cells. This finding is in accordance with what is previously observed in *in vitro* and *in vivo* experiments (Dobora et al., 1995; Andersen et al., 1994). The osmolality appears to play a role, with the IOCM being most extensively internalized followed by the three LOCM. However, the cellular mechanism for this is not known. The concentration of ICM within the vacuoles it is not known, and it may be that even though the cells exposed to iodixanol showed the most pronounced vacuolisation they may contain lower concentrations of ICM compared to the cells exposed to LOCM.

Therefore, the findings from the two methods used to evaluate uptake of ICM in NRK 52-E cells are partly contradictory, and more complex confirmatory experiments are needed to reassess these findings.

5.7 NRK 52-E vs. MDCK and LLC PK-1 cells

The cell line most frequently used to evaluate toxic effects of ICM *in vitro* are MDCK cells, which display features of a distal tubular cell phenotype. However, the proximal tubules are the region of the kidneys where necrosis is most commonly found after administration of ICM in laboratory animals (Tumlin et al., 2006). Therefore, the choice of a cell line of proximal origin (NRK 52-E) seemed the most relevant. In addition to the origin in the kidney, the rat is the species most frequently utilized for *in vivo* experiments, and therefore a cell line with rat origin was chosen.

The results from the *in vitro* experiments strongly indicate that IOCM are less toxic than LOCM. This indicates one of two possibilities; either the experimental procedures were optimised sufficient to detect differences between the four ICM, or alternatively the NRK 52-E cells might be more susceptible to LOCM over IOCM compared to MDCK and LLC PK-1 cells.

5.8 The rat-gentamicin model

5.8.1 Refinement of the rat-gentamicin model

Several studies have used a rat-gentamicin model to evaluate renal toxicity of ICM (Idee et al., 1995;Thomsen et al., 1990b;Thomsen et al., 1991;Thomsen et al., 1993;Uchimoto et al., 1994). However, these models have limitations and disadvantages. In previous studies, gentamicin has been injected daily for up to 23 days (Thomsen et al., 1990b;Thomsen et al., 1993;Uchimoto et al., 1994)), and this may be criticised since the toxic effects of gentamicin start to recover after approximately 10 days (Gilbert et al., 1979).

The model developed in this thesis has several advantages. Due to injection over four consecutive days and euthanizing on the fifth day, the experiment can be completed within one working week. The use of metabolism cages on the urine refrigerated rack facilitates the collection of urine samples of good quality compared to manual sampling in metabolism cages. The dose of gentamicin is administered s.c., an administration route which is less

traumatic for the animals compared to other administration routes (e.g. i.v. or i.m.). S.c. administration of gentamicin has previously been described in the literature (Pedraza-Chaverri et al., 2004). Most of the authors using the rat-gentamicin models evaluate ICM toxicity has administered gentamicin through the i.m. route (Idee et al., 1995;Thomsen et al., 1990b;Thomsen et al., 1993;Uchimoto et al., 1994), while one study has reported i.v. administration of gentamicin (Thomsen et al., 1991).

Furthermore, the induced renal impairment caused by gentamicin alone seems to be dependent on the dose level. The dose selected as the definitive dose in the rat-gentamicin model was 70 mg/kg bw. The histopathological findings in this group revealed that the kidneys were impaired but not severely damaged, which is in accordance with the aim of the study. A severely damaged kidney would have made it more difficult to differentiate between the damage caused by gentamicin alone and the damage caused by ioversol.

5.8.2 Average weight during the study

The rats showed no signs of discomfort during the study. All groups had similar weight gains compared to the saline group, with no overt adverse clinical signs. In some of the previously reported rat-gentamicin models which have evaluated ICM, the weight curve of the animals has either gone down (Idee et al., 1995;Thomsen et al., 1991), or been highly variable (Thomsen et al., 1990b;Thomsen et al., 1993). A highly variable weight curve creates an uncertain model and could indicate a higher level of stress for the animals. Therefore, it can be concluded that the well-being of the rats were maintained throughout the present study.

5.8.3 Gross pathology

Gross pathology examination revealed that the kidneys of the animals in the treatment groups were pale compared to the control group. The paleness increased with the administered dose of gentamicin and was most pronounced in the gentamicin-ioversol group. One explanation of the paleness of the kidneys is that the administered dose of gentamicin induces reduced blood flow and some ischemic damage secondary to the gentamicin damage. This observation suggests that actually two factors contribute to the pathogenesis that is induced by administration of one nephrotoxic substance.

The fact that gross kidney lesions were only detected in the gentamicin-ioversol group indicate that there is a major difference in the renal damage induced by gentamicin treatment alone and gentamicin treatment followed by ICM administration. Hence, the aim to make rats susceptible to ICM was accomplished. Furthermore, the finding confirms that the rat-gentamicin model alone has a relatively low toxicity to the kidneys, making it possible to detect toxicity induced by ICM.

5.8.4 Increase in kidney weight

Statistically significant changes were found between the control group and the treatment groups when kidney weight in percent of total body weight was compared (relative kidney weight). Most likely, the increase in kidney weight was not a consequence of hypertrophy to compensate for the decreased renal function, but rather a consequence of oedema. The histopathological changes detected within one experimental group was very consistent, and the same might be the case with oedema as the increase in kidney weight was so consistent, and so little variability was found within each group. Overall, the minor weight changes were considered to have no major toxicological significance.

5.8.5 Histopathology

Several of the previously reported rat-gentamicin models evaluating kidney damage caused by ICM have included histopathology as an endpoint (Idee et al., 1995;Thomsen et al., 1990b;Thomsen et al., 1991;Thomsen et al., 1993). The histological featured evaluated include dilated tubules, necrotic tubules, interstitial inflammation (Thomsen et al., 1990b;Thomsen et al., 1991;Thomsen et al., 1993), vacuolation, tubular lesions, necrosis, and vascular lesions (Idee et al., 1995).

Histopathology features after gentamicin treatment

One purpose of this present rat-gentamicin model was to create a model which impaired the renal function without causing any pronounced damage to the kidneys. A model like this is more representative for patients at risk compared to models with severe renal damage. In addition it makes it easier to differentiate between the nephrotoxic damage induced by gentamicin alone and the damage induced by ICM.

In this present work the gentamicin-related histopathological findings were noted in the kidneys at all doses. Hyaline droplet formation in the proximal tubular epithelium was observed in most of the animals at all doses, the effected tubules were present throughout the outer cortex, and the severity was dose-related and ranged from minimal to marked. Interstitial inflammation was present to al less extent (3 rats in total), and the severity ranged from minimal to mild. Necrosis, vacuolisation, and tubular dilatation were not noted in any of the gentamicin treated animals. Since no severe kidney damage was induced, the goal to create an improved model was achieved.

Idée et al. (1995) administered gentamicin (50 mg/kg bw, i.m.) for 4 consecutive days, and is therefore comparable to the one developed in this present work. However, Idée et al. had a more complex model as they used nephrectomized rats (one kidney removed) and clamped the blood supply to the remaining kidney 3 minutes prior to ICM administration. The histopathological findings in gentamicin treated rats included vacuolisation (moderate size, sometimes containing a dense inclusion) of the epithelial cells of the convoluted tubules. Furthermore, some tubular lesions and limited focal inflammation were observed in a few of the gentamicin treated animals.

Not surprisingly, the rat-gentamicin models which injected gentamicin for a prolonged period observed more pronounced histopathological changes than in the present model. Thomsen et al (1990b) reported that gentamicin treatment (40 mg/kg bw i.m. for up to 23 consecutive days) induced dilated tubules, minor to moderate interstitial inflammation, and necrosis. In another report by Thomsen et al. (1991) severe tubular necrosis and interstitial inflammation were found after 12 days of gentamicin treatment (40 mg/kg bw i.v.) and minimal necrosis, unchanged inflammation, and some tubular dilatation after 18 days of gentamicin treatment (40 mg/kg bw i.v.). In their most recent rat-gentamicin study, Thomsen et al. (1993) it was reported that gentamicin treatment (40 mg/kg bw i.m. for 17 consecutive days) caused slightly dilated tubules, moderate necrosis, and slightly interstitial inflammation.

Histopathology features after ICM administration

In this present work the gentamicin-ioversol treatment group was associated with histopathological findings in the kidney that consisted of minimal to severe hyaline droplet formation, minimal to moderate tubular vacuolation, minimal to marked tubular dilatation,

minimal to severe tubular necrosis, and minimal to severe interstitial inflammation. In all cases the tubular lesions were present in the proximal tubules and all lesions were noted in the outer cortex of the kidney only. These findings are in accordance with previously findings in rat-gentamicin studies evaluating the nephrotoxic effect of ICM (see following sections). Several of the previously reported models have failed to show a pronounced difference between the kidney damage induced by gentamicin treatment alone, and by gentamicin-ICM treatment. However, this was fully accomplished in the present work. One explanation to why this was achieved is that the gentamicin induced kidney damage in this study was minor compared to several of the previously reported models.

The observed tissue alterations after administration of ioversol in this present rat-gentamicin model, as examined by histopahthology, are somewhat similar to what is observed in CIN patients. Interstitial inflammation, vacuolation, tubular atrophy and/or necrosis, are among the features which can be seen both in patients (Thomsen and Morcos, 2003;Tumlin et al., 2006) and in this present model.

The rat-gentamicin model reported by Idee et al. (1995) is, as mentioned, the model that is the most similar to the one developed in this present thesis. The histological changes observed in the groups receiving ICM included more severe necrosis and vascular lesions compared to the gentamicin group. Such changes were seen in the kidneys of rats administered with diatrizoate alone or gentamicin and diatrozaote, but were not present in the groups receiving ioxaglate alone or gentamicin and ioxaglate.

Thomsen et al. (1990b) reported histological changes following administration of diatrizoate and iohexol (both at a concentration at 1.75 g I/kg) in their rat-gentamicin model (kidneys were removed 3 and 6 days after administration of ICM). The features detected in the diatrizoate group were the same as in the gentamicin group and included dilated tubules, minor to moderate interstitial inflammation, and necrosis in a few of the animals. No differences were detected between the groups where the kidneys were taken 3 days and 6 days after administration of ICM. In the iohexol group, the histopathological changes were the same as in the diatrizoate group, although more pronounced in the kidneys taken 3 days after administration of ICM. In the kidneys taken 6 days after iohexol administration, no differences were seen between the two groups. The findings in the study indicated that

iohexol had an additional direct toxic effect on the kidneys in the rat-gentamicin model, whereas diatrizoate had no such effect. In another rat-gentamicin study by Thomsen et al. (1991), the nephrotoxic effect of diatrizoate and iohexol were compared and histopathology was included as an endpoint of toxicity. The ICM were injected after 9 days of gentamicin administration, and the kidneys were taken out 3 and 6 days after ICM administration. The kidneys taken 3 days after diatrizoate administration revealed histological changes identical to the gentamicin group. The changes included interstitial inflammation, severe necrosis, as well as some dilated tubular profiles in the cortex. In the kidneys taken out 6 days after administration of diatrizoate, the interstitial inflammation was unchanged, almost no necrosis was present, and more dilated tubular profiles were found. In the iohexol group, the histology findings 3 days after iohexol administration was the same as those found in the saline and the diatrizoate group. In the kidneys taken out 6 days after iohexol administration, focal necrosis was observed, but not as many as seen on day 12. Furthermore, dilatation of several tubular profiles were observed in the cortex and medulla, and interstitial inflammation was more pronounced than in the diatrizoate group. This finding indicated that iohexol had an additional nephrotoxic effect. In their most recent rat-gentamicin study, Thomsen et al. (1993) reported histopathological features following administration of different doses of iohexol. The features observed included slightly to moderate diluted tubules and slightly to moderate tubular necrosis. Both of these features were about the same as in the gentamicin group, indicating that iohexol had no further deleterious effect on the kidneys. Furthermore, they reported a more pronounced interstitial inflammation compared to the gentamicin group, and concluded that iohexol might be responsible for this.

It is surprisingly that Thomsen et al. (1990b;1991) reported an additional nephrotoxic effect caused by iohexol, but not diatrizoate, as diatrizoate has a higher osmolality than iohexol. However, in both models, gentamicin was administered for a prolonged period (23, 12 and 18 days) and severe histopathological changes, compared to what was observed in this present model, were induced by gentamicin treatment. The severe damage caused by gentamicin treatment in these studies complicates the discrimination of effects between gentamicin and ICM.

5.9 Biomarkers of renal injury

5.9.1 Creatinine

Creatinine clearance is considered to be more accurate than serum creatinine to evaluate renal function (Idee and Bonnemain, 1996). To calculate creatinine clearance properly, timed urine and serum samples are needed. In this study the time between the urine sample on day 4 and blood sample on day 5 was too large to calculate creatinine clearance accurately, therefore serum creatinine was included as a separate biomarker. As mentioned, approximately 50 % of renal function must be lost before any statistically significant rise in serum creatinine occurs. Hence, serum creatinine is not an early marker or a very sensitive marker of renal injury.

Creatinine has several disadvantages as a renal biomarker. It has been shown to increase in diseases that do not directly affect the renal blood flow or GFR (Sansoe et al., 2002). Serum levels might be elevated in individuals with a high meat diet, with increased muscle mass, or after prolonged exercise or acute muscle damage. Consequently, serum creatinine will be lower in those who have undergone loss of muscle mass (Hart and Kinter, 2005). However, these limitations are more important in a clinical situation than in laboratory animals where most of the influences mentioned are under control.

Diurnal variations in serum creatinine has been detected in male and female Sprague Dawley rats, with serum creatinine levels being the lowest at the onset of the dark period and the highest at the end of the dark period (Minematsu et al., 1995). The urine samples were collected at the same time point throughout the present study (09.00 to 21.00), so the diurnal variation should not be a source of variation.

The present results indicate that neither serum creatinine nor urine creatinine are useful as biomarkers for renal damage in the present rat-gentamicin model. Serum creatinine results showed high variability within each group. Taken together this indicates that the variation from day to day is relatively high and that serum creatinine is not useful as a biomarker of renal damage caused by ICM in the present rat-gentamicin model. The mean total urinary creatinine excretion increased in each group, and the variation was less pronounced than in serum. No statistically significant differences were found between the groups. Hence, urine

creatinine is probably not a useful biomarker of ICM renal damage in this present model.

However, both urine and serum creatinine should be included in the rat-gentamicin model for two reasons: (1) To serve as a control for early biomarkers; if the evaluated biomarker is positive while serum creatinine is negative, it can be concluded that it is a more sensitive and an earlier biomarker of renal injury than serum creatinine; (2) to normalize other urinary parameters to urinary creatinine, which is the most frequently used approach to normalize urine parameters.

5.9.2 Cystatin C

Cystatin C was measured with an ELISA kit with anti human cystatin C since there is no commercially available ELISA kit for rat cystatin C. Some cross reactivity between the human antibody and rat antigen is observed, and the supplier reports this cross reactivity with rat serum giving a 22 % signal of human serum (no reported value for urine). At least one rat nephrotoxicity study has used the human cystatin C antibodies to detect rat cystatin C (Bökenkamp et al., 2001), which was the reason to include it in this present work. All urine and serum samples were, however, below the detection limit of the kit (200 ng/ml). The rat-gentamicin model on its own and combined with administration of ioversol did not induce enough renal damage to give a rise in cystatin C concentrations which were detectable with a human ELISA kit.

5.9.3 Total urinary protein

One of the clinical manifestations of CIN is a mild and transient proteinuria (Morcos and Thomsen, 2001;Thomsen and Morcos, 2003). Proteinuria has previously been measured as both urinary total protein (Uchimoto et al., 1994) and albumin (Thomsen et al., 1990b;Thomsen et al., 1991;Thomsen et al., 1993), and used as a biomarker of nephrotoxicity in the rat-gentamicin model. Ushimoto et al. (1994) showed that the excretion of protein was not affected by administration of ICM, whereas all the rat-gentamicin studies reported by Thomsen et al. (1990b;1991;1993) have shown an increase in urinary albumin after administration of ICM. The results in the present work suggest that total urinary protein excretion is suitable as a biomarker of nephrotoxic effects of ICM, as only the gentamicin-ioversol group showed to be statistically significant higher than the control.

5.9.4 N-acetyl-β-glucosaminidase

NAG has previously been reported as a sensitive parameter for the early assessment of renal effects of ICM in patients (Duan et al., 1999), and NAG has been frequently used as a marker of ICM effects in rat-gentamicin models (Idee et al., 1995;Thomsen et al., 1990b;Thomsen et al., 1991;Thomsen et al., 1993;Uchimoto et al., 1994), in normal rat models (Thomsen et al., 1988;Thomsen et al., 1994), and in other rat models (Beaufils et al., 1995;Thomsen et al., 1990a). In rat-gentamicin studies, the NAG results have been conflicting. Ushimoto et al. (1994) and Thomsen et al. (1990b;1991) reported an increase in urinary NAG activity after administration of ICM compared to the control group, whereas Thomsen et al. (1993) and Idée et al. (1995) reported no effect in NAG activity after administration of ICM.

In this present work it was found that all treatment groups had a statistically significantly higher increase in NAG activity compared to the control group (saline), indicating that both gentamicin and ioversol are responsible for the increase in urinary NAG activity. Urinary NAG activity was found to increase with the administered dose of gentamicin, and was the most pronounced in the gentamicin-ioversol group. No statistically significant difference was found between the gentamicin 70 mg/kg bw group and the gentamicin-ioversol group, despite the marked difference in means (114 % and 144 % respectively). Therefore, it is not known whether the increase in urinary NAG activity was due to the effect of gentamicin, or if ioversol also plays a role. Based on their difference in means it seems fair to conclude that NAG may be a promising biomarker for nephrotoxic effects after administration of ICM. However, a follow up study should be performed to verify the result and to decide if the gentamicin alone or ICM is responsible for the increase in urinary NAG activity.

NAG has several properties facilitating its use as a marker of renal toxicity. Its molecular size (150 kDa) preclude glomerular filtration, thus urinary NAG is a result of excretion from the kidneys. NAG is stable in urine compared to other enzymes, and can be stored in the freezer for several months without degradation occurring (Price, 1992). However, the use of NAG is limited by the fact that urinary excretion of the enzyme is also elevated in glomerular diseases such as diabetic nephropathy (Trof et al., 2006). This is not a problem in *in vivo* experiments with non-diabetic animals.

5.9.5 γ-Glutamyl transferase

GGT has previously been stated to be a sensitive parameter for the early assessment of subclinical nephrotoxicity induced by ICM in patients (Duan et al., 1999). It has previously been reported as a biomarker of nephrotoxicity of ICM in rat-gentamicin models (Thomsen et al., 1990b;Thomsen et al., 1991;Thomsen et al., 1993;Uchimoto et al., 1994), in normal rat models (Thomsen et al., 1988;Thomsen et al., 1994), and in other rat models (Thomsen et al., 1990a). An increase in urinary GGT activity was reported after administration of ICM in all rat-gentamicin models reported (Thomsen et al., 1990b;Thomsen et al., 1991;Thomsen et al., 1993;Uchimoto et al., 1994), and in one of them, a dose-dependent effect of iohexol was observed (Thomsen et al., 1993).

In this present work, the increase in urinary GGT activity in the gentamicin-ioversol group was statistically significantly higher than in the control group, whereas no other statistically significant differences were detected. This finding suggests that GGT is suitable as a biomarker for nephrotoxicity after administration of ICM in this present rat-gentamicin model.

Limitations exist with the use of GGT as a marker of nephrotoxicity. GGT is somewhat unstable in urine, and no stabilizers seem to prevent degradation (Hart and Kinter, 2005). Therefore, analysis should be assessed as quickly as possible after sample collection. Matteucci et al. (1991) showed that GGT is partly preserved at -70ºC only if the sample has been previously centrifuged. In this study it was not possible to analyse all samples as they were collected, thus the samples were centrifuged and frozen down at -70ºC until analysis. Analysis was performed approximately 1 to 2 months after collection. A follow-up study with analyses of GGT performed shortly after collection should be performed to reassess the results. In previous rat-gentamicin models with ICM administration, urine samples have mostly been frozen down for later analysis (-20ºC) (Thomsen et al., 1990b;Thomsen et al., 1991;Thomsen et al., 1993), while only one study analysed the urine samples immediately after collection (Uchimoto et al., 1994).

5.9.6 α-Glutathione S-transferase

To my knowledge, only one clinical study has included α-GST to assess renal tubular toxicity

caused by ICM administration, whereas no animal studies have included α-GST. Arici et al. (2003) found that urinary levels in patients increased after administration of ICM, not returning to baseline levels before the 7 days after administration. This study demonstrates that α-GST might be a useful clinical marker of nephrotoxicity.

In this present work no statistically significant differences between the control group and the treatment groups were detected, however large differences in mean values in the groups were observed, with the gentamicin-ioversol showing the most pronounced increase in urinary α-GST excretion. Since the purpose was to elucidate biomarkers of CIN, and due to the high cost of the kit, the samples were not analysed in duplicate or triplicate as recommended. If the samples had been analysed in triplicate it might have been possible to detect and omit outliers, and thus the mean would possibly have been more representative and the variation minimised.

Overall, α-GST might be promising as a biomarker of renal damage caused by ICM in the present model. A follow-up study where the samples are analysed in triplicate would possibly give more representative results and make it possible to draw any conclusion about α-GST as a biomarker of renal damage induced by ICM.

5.9.7 Urine cytology

Despite the many years of research, relatively few studies evaluating renal toxicity of ICM have included urine cytology as a marker of renal damage. However, urine sediment content can provide information of the real damage and should be included as a marker of renal damage. Previously, the utility of urine cytology has been limited by temporary wet preparations that have to be examined shortly after collection. However, the use of fixed Papanicolaou stained preparations facilitate the use of urine cytology as a marker of renal toxicity. Stetinova et al. (1996) compared the minute excretion of epithelial cells in urine to excretion of NAG and alanine aminopeptidiase (AAP) after administration of the nephrotoxins amphotericin B and cyclosporine A, and found that the increase in excretion of epithelial cells was more suitable to detect nephrotoxicity than NAG and AAP.

In the present thesis, microscopic evaluations were preformed on both fresh and formalin fixed urine sediment (same sample) to assess if the two approaches gave the same results. The

same results were seen, and based on that finding, urine cytology was assessed on formalin fixed preparations throughout the study.

Under normal physiological conditions, about 50 flat epithelial cells from the urinary bladder per minute can be found in the urine (Stetinova et al., 1996). In the present work it was demonstrated that both the gentamicin treatment and administration of ioversol was responsible for renal toxicity as more than 50 epithelial cells per minute were detected for all treatment groups. The number of epithelial cells per minute increased with the administered dose of gentamicin and was most pronounced in the gentamicin-ioversol group. Large differences, although not statistically significant, were detected between the gentamicin 70 mg/kg bw and the gentamicin-ioversol group. These findings suggest that the number of epithelial cells per minute can be used as a reliable marker of renal damage in the present rat-gentamicin model. However, a follow-up study should be performed to evaluate whether gentamicin treatment alone or co-administration of ioversol is responsible for the sloughing of cells into the lumen of the tubule.

5.9.8 Conclusion of the rat-gentamicin model and the biomarkers evaluated

The present rat-gentamicin model was for the first time tested as an *in vivo* animal model of renal damage at GE Healthcare, and both the histopathological findings and the biomarker findings suggest that the model may be a useful model for testing of ICM. However, new experiments should be performed to validate the model further.

Due to the time course of this master's thesis only one comprehensive study was conducted to elucidate biomarkers of renal damage caused by ICM in the rat-gentamicin model. A follow-up study should be performed to confirm the findings in this present study, and to evaluate the promising biomarkers further. This is needed for three reasons; (1) the biomarkers that showed to be useful as biomarkers of renal damage induced by administration of ioversol in the rat-gentamicin model were both of non-normal distribution due to overall high variance in the groups, and a follow up study is needed to see if the variation remains high, and if the findings are the same in normal distributed settings; (2) to elucidate the promising biomarkers to reassess if they are useful as biomarkers of renal damage cause by ICM in the present rat-gentamicin model; (3) to discriminate the effects of different ICM.

Creatinine did not prove to be a useful marker of renal damage caused by ICM in this present rat-gentamicin model, however, the parameter should be included to possibly distinguish early and late markers of injury, in addition to be used for normalization of other urine parameters. NAG, α-GST, and urine cytology showed to be promising biomarkers of renal damage in the present rat-gentamicin model, but further experiments are needed to confirm this. Total protein and GGT were the only biomarkers evaluated that proved to be suitable for the induced renal damage by ICM in the rat-gentamicin model.

The use of clinical chemistry parameters (GGT, NAG, total protein) are beneficial for several reasons; first, it is easy and relatively inexpensive to measure these parameters on a clinical chemistry analyser compared to using ELISA kit; second, none of them requires a stabilisation buffer, and hence that facilitate the urine sampling. Furthermore, these biomarkers are validated as a sensitive biomarker for renal injury in both rat and human, and hence it is possible to use the same biomarker in *in vitro* experiments, animal experiments and clinical trials.

5.10 Conclusion

In cultured cells of rat proximal tubule origin (NRK 52-E cells) the iso-osmolal contrast medium iodixanol was found to be less toxic than low-osmolal contrast media iopromide, ioversol, and iohexol. The osmolality of the contrast media was shown to correlate with the observed toxicity. In addition, the chemical structure of the contrast agents may also contribute to the toxicity in NRK 52-E cells.

Histopathological changes, although not present in the gentamicin only treatment groups, were detected in the gentamicin-ioversol group. Furthermore, several of the biomarkers were elevated in the gentamicin-ioversol group compared to the gentamicin groups. Overall, these findings show that the present rat-gentamicin model might be a good animal model for evaluating the nephrotoxic effects of ICM, and possibly to evaluate novel contrast agents.

Total protein and GGT were shown to be suitable biomarkers of renal damage caused by ioversol in the rat-gentamicin model, while α-GST, NAG, and urine cytology were also promising markers which need further evaluation.

Proposal for further studies

The *in vitro* system used to evaluate the physiochemical properties of ICM showed marked differences between IOCM and LOCM. The finding that IOCM are better tolerated than LOCM are in conformity with what has been observed in patients (Aspelin et al., 2003). It is not known whether the NRK 52-E cell line is more susceptible to LOCM than other cell lines, or if the optimized methods used in this work were superior to the ones used in previously reported studies. It would be of interest to use the same methods as presented in this thesis to evaluate ICM toxicity in the cell lines most frequently used in this field (MDCK and LLC PK 1).

Further studies should be performed on the rat-gentamicin model for several purposes: (1) The model should be validated as a model of renal damage caused by ICM based on the criteria given in the introduction; (2) a gentamicin 70 mg/kg bw group and a gentamicin-ioversol group should be compared to assess if the promising biomarkers are biomarkers of gentamicin nephrotoxicity or of ICM nephrotoxicity; (3) a saline-ICM group should be included and be compared to the a gentamicin-ICM group to evaluate the utility of gentamicin to sensitize the rats; (4) several new and promising marker of renal damage exist, and kidney injury molecule-1 (KIM-1) (Han et al., 2002;Vaidya et al., 2006;van Timmeren et al., 2006), osteopontin (Verstrepen et al., 2001), cysteine-rich protein 61 (CYR-61) (Muramatsu et al., 2002) and neutrophil gelatinase-associated lipocalin (NGAL) (Mishra et al., 2004;Mishra et al., 2003;Mishra et al., 2006) are among the novel biomarkers that should be elucidated as markers of nephrotoxicity caused by ICM in the present rat-gentamicin model.

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Appendix 1

Table 14 The iodinated x-ray contrast media discussed throughout this thesis

Contrast medium	**Classification**	**Osmolality (mOsm/ kg H_2O)**
Diatrizoate	Ionic monomer (HOCM)	1270-1550 (300 mg I/ml)
Iohexol	Non-ionic monomer (LOCM)	640 (300 mg I/ml)
Iodixanol	Non-ionic dimer (IOCM)	290 (320 mg I/ml)
Iopromide	Non-ionic monomer (LOCM)	610-620 (300 mg I/ml)
Ioversol	Non-ionic monomer (LOCM)	630 (300 mg I/ml)
Ioxaglate	Ionic-dimer (LOCM)	600 (320 mg I/ml)
Iopamidol	Non-ionic monomer (LOCM)	616-680 (300 mg I/ml)
Iotrolan	Non-ionic dimer (IOCM)	270 (240 mg I/ml)
Iomeprol	Non-ionic monomer (LOCM)	521-609 (300 mg I/ml)

Appendix 2

Normal distribution and equal variance was assumed for the results of the trypan blue exclusion assay after 12 and 24 hours exposure to controls and ICM.

Table 15 and *Figure 20* shows the non-parametric statistical analysis after 12 hours exposure to controls and ICM.

Table 15 Percent cell death measure by the trypan blue exclusion assay after 12 hours exposure to control, iodixanol, iohexol, iopromide, and ioversol

	Max	Min	Median	25 %	75 %	p < 0,05 *
Control	8.00	4.00	6.00	5.50	7.00	
Iodixanol	15.00	8.00	12.00	8.75	13.50	No
Iohexol	60.00	30.00	52.00	40.50	57.75	Yes
Iopromide	40.00	35.00	37.00	35.75	38.50	Yes
Ioversol	46.00	37.00	43.00	41.50	43.75	Yes

** indicates significant difference relative to the control group (Kruskal-Wallis ANOVA on ranks with Dunn's post hoc test. n = 5 for the treatment groups and 20 for the control group. p < 0.05).*

When the four ICM were compared pairwise no statistically significant differences was found.

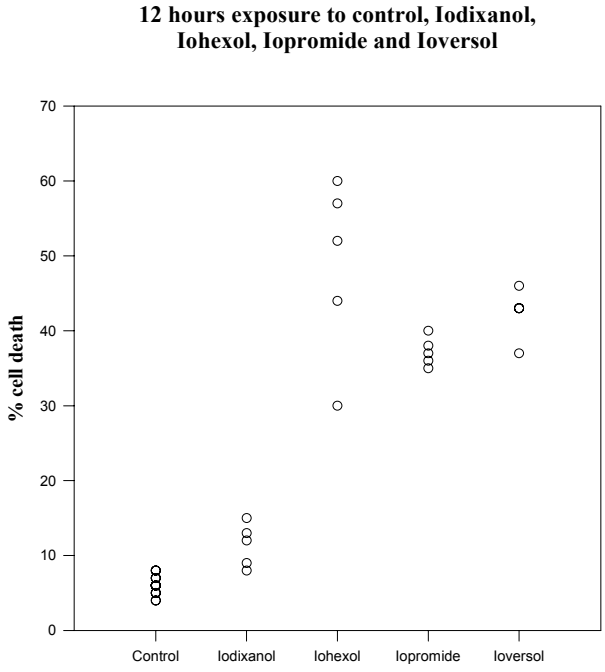

Figure 20 Percent cell death after exposure to control, iodixanol, iohexol, iopromide, and ioversol (150 mg I/ml) for 12 hours. Each dot represents one independent experiment.

Table 16 and *Figure 21* shows non-parametrical analysis of the result from the trypan blue exclusion assay after 24 hours exposure.

Table 16 Percent cell death measured by the trypan blue exclusion assay after 24 hours exposure to control, iodixanol, iohexol, iopromide, and ioversol

	Max	Min	Median	25 %	75 %	p < 0,05 *
Control	14.00	4.00	7.00	6.00	9.50	
Iodixanol	41.00	27.00	32.00	29.25	38.75	No
Iohexol	87.00	78.00	81.00	78.75	83.25	Yes
Iopromide	69.00	40.00	56.00	44.50	65.25	Yes
Ioversol	64.00	51.00	56.00	53.25	63.25	Yes

** indicates significant difference relative to the control group (Kruskal-Wallis ANOVA on ranks with Dunn's post hoc test. n = 5 for the treatment groups and 20 for the control group. p < 0.05).*

When the four ICM were compared pairwise no statistically significant differences was found.

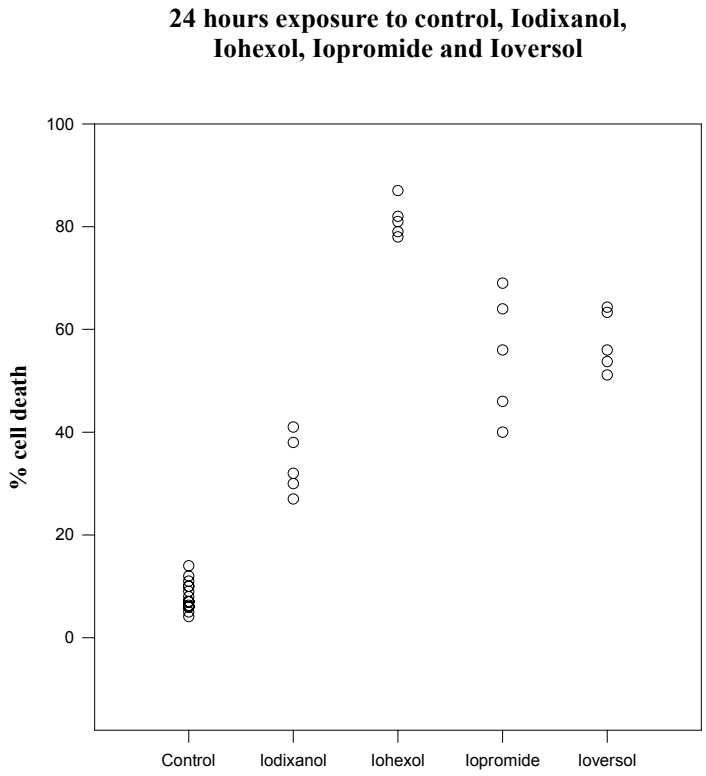

Figure 21 Percent cell death measured after 24 hours exposure to control, iodixanol, iohexol, iopromide, and ioversol (150 mg I/ ml). Each dot represents one replicate.