In vitro assessment of primary human chondrocyte viability following treatment with intra-articular contrast agents and local anaesthetic

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Masters in Surgery (MCh.)

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Misericordia

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Declaration

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List of Abbreviations

ACI	Autologous Chondrocyte Implantation
ACL	Anterior Cruciate Ligament
ATP	Adenosine Triphosphate
CI	Confidence Interval
CIN	Contrast-Induced Nephropathy
CKD	Chronic Kidney Disease
CT	Computed Tomography
Ct	Cycle Threshold
dd H_2O	Double Distilled Water
DDH	Developmental Dysplasia of the Hip
DEPC	Diethyl Pyrocarbonate
cDNA	Complementary Deoxyribonucleic Acid
DNA	Deoxyribonucleic Acid
DMEM	Dulbecco's Modified Eagle's Medium
dNTP	Deoxynucleoside Triphosphate
dT	Deoxythymine
ECM	Extracellular Matrix
EDTA	Ethylenediaminetetraacetic Acid
GAG	Glycosaminoglycan
GAPDH	Glyceraldehyde 3-phosphate Dehydrogenase
Gd	Gadolinium
Gd-DOTA	Gadolinium Tetra-azacyclododecane Tetraacetic Acid

Gd-DTPA	Gadolinium Diethylenetriamine Pentaacetic Acid
HEPA	High Efficiency Particulate Air
HOCM	High Osmolar Contrast Media
Il-6	Interleukin-6
IOCM	Intermediate Osmolar Contrast Media
LDH	Lactate Dehydrogenase
LOCM	Low Osmolar Contrast Media
MMUH	Mater Misericordiae University Hospital
MR	Magnetic Resonance
MRI	Magnetic Resonance Imaging
MTS	3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium)
MTT	3-(4, 5-dimethylthiazol-2-yl)-2, 5-dephenyl tetrazolium bromide
NSF	Nephrogenic systemic fibrosis
PAGCL	Post-Arthroscopic Glenohumeral Chondrolysis
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PES	Phenazine Ethosulfate
PHC	Primary Human Chondrocyte
RNA	Ribonucleic Acid
RNase	Ribonuclease
RPM	Revolutions per Minute
RT	Real Time
SD	Standard Deviation

SE	Standard Error
THA	Total Hip Arthroplasty
TKA	Total Knee Arthroplasty
TRI	Total RNA Isolation
TUNEL	Tdt-mediated dUTP neck-end labeling
µL	Microliter
µm	Micrometre
V: V	Volume: Volume

Summary

Title

In vitro assessment of primary human chondrocyte viability following treatment with intra-articular contrast agents and local anaesthetic.

Introduction

Magnetic resonance (MR) and computed tomography (CT) direct arthrography, procedures in which either gadolinium or iodine-based contrast agents are administered intra-articularly, are commonly used tools in musculoskeletal diagnosis. Although considered safe for systemic use, toxicities have been identified for these agents in some tissues, yet, there have been few studies examining their toxicity on the joint. The aims of this study were to (i) investigate the effect of four routinely used intra-articular contrast agents on primary human chondrocyte viability in both a time-dependent and dose-dependent fashion, and (ii) determine the additive effect of local anaesthetic to the contrast media.

Methods

Primary human chondrocytes, isolated from 34 femoral heads obtained at the time of bipolar hemiarthroplasty for femoral neck fracture, were grown in monolayer culture. Chondrocyte phenotype was confirmed with Collagen II expression. In one set of experiments, chondrocytes were exposed to each contrast agent in isolation (Iohexol 50, 100, 200 mg/ml; Iopromide 50, 100, 200 mg/ml; Gadopentetate Dimeglumine 1.7, 2.5, 5, 10 mmol/L; Gadobenate Dimeglumine 1.7, 2.5, 5, 10 mmol/L) for 1, 4, 16 or 24 hours. Each agent was in solution with cell culture media. In a second set of experiments, chondrocytes were exposed to a clinical injection 'cocktail' (Iohexol 150 mg/ml; Gadopentetate Dimeglumine 2.5 mmol/L; Lidocaine 0.5%; Lidocaine 1%; Iohexol + Lidocaine [final concentrations of 150 mg/ml and 0.5%, respectively]; Gadopentetate Dimeglumine + Lidocaine [final concentrations of 2.5 mmol/L and 0.5%, respectively]) for 30, 45 and 60 minutes. Cells exposed to cell culture media or saline served as controls. Twenty-four hours after exposure, cell viability was assessed using the Cell-Titer 96® AQueous One Solution Cell Proliferation Assay and crystal violet staining.

Results

Gadolinium-based contrast agents did not cause a decrease in chondrocyte viability in either a time-dependent or dose-dependent fashion. At 1 and 4 hours, iodine-based contrast agents did not cause a decrease in chondrocyte viability at any concentration. At 16 hours, a significant decrease in cell viability was noted at both 100 mg/ml and 200 mg/ml in the Iohexol-treated group and at 200 mg/ml in the Iopromide-treated group. At 24 hours, no decrease in cell viability was noted at any concentration in the Iohexol-treated group, but cell viability was reduced at 100 mg/ml and 200 mg/ml in the Iopromide-treated group. Exposure of primary human chondrocytes to any concentration of lidocaine - either in isolation or in solution with Iohexol or Gadopentetate Dimeglumine - led to a profound decrease in cell viability at each time point.

Conclusion

Gadolinium-based contrast agents are safe for intra-articular injection at the concentrations routinely used in clinical practice. While prolonged exposure to higher doses of iodine-based contrast agents led to a decrease in cell viability in comparison to a media control, no evidence of a time-dependent effect was observed at a concentration of either 50 mg/ml or 200 mg/ml. The addition of lidocaine to both MR and CT arthrogram solutions caused a significant decrease in cell viability, which occurred in both a time-dependent and dose-dependent fashion. These *in vitro* findings suggest that local anaesthetics should not be routinely added to intra-articular radiographic contrast agents.

Chapter 1

Introduction

1.1 The typical synovial joint

1.1.1 Anatomy of a typical synovial joint

Typical synovial joints, which include all limb joints, are characterized by six features: the bone ends are covered by *hyaline cartilage* and are surrounded by a *capsule* which encloses a joint *cavity*. The capsule is reinforced externally, internally or both by *ligaments* and is lined internally by a *synovial membrane*. The joint is capable of varying degrees of *movement* (1). In atypical synovial joints, the articular surfaces are covered by fibrocartilage.

The synovial membrane lines the capsule and invests all non-articulating surfaces within the joint, finding attachment to the articular margin of each bone. Cells of the membrane produce a hyaluronic acid derivative which is responsible for maintaining the viscosity of the fluid, whose main function is lubrication. In normal joints the fluid is a mere film. The largest joint, the knee, only contains 0.5 ml.

A ring of fibrocartilage, the labrum, is attached to the margins of the articular cavity of certain joints (e.g. the shoulder and hip joints). This serves to slightly, but effectively, deepen the articular cavity.

Intra-articular fibrocartilages, discs or menisci, in which the fibrous element is predominant, are found in certain joints. They may be complete, dividing the joint cavity into two, or incomplete. They occur characteristically in joints in which the congruity between the articular surfaces is low (e.g. the knee joint)(1).

Fatty pads are found in some synovial joints, occupying spaces where bony surfaces are incongruous. The Haversian fat pad of the hip joint and the patellar fat pad of the knee joint are examples.

1.1.2 Articular cartilage

Hyaline cartilage coats the articular surfaces of typical synovial joints and is composed of individual chondrocytes bound together by an extracellular matrix (ECM). The function of hyaline cartilage is to distribute weight-bearing forces and reduce friction. It is avascular, aneural, alymphatic and almost non-immunogenic (2). It is nourished entirely via diffusion from the synovial fluid.

The structure of cartilage can be divided into layers as seen on histological examination (3). Zone 1 is the superficial or gliding zone. This separates the cartilage from the surrounding

tissue or fluid. This zone, the so-called *lamina splendens*, consists of a clear film of small collagen fibrils and a cellular layer of flattened chondrocytes one to three cells thick. The collagen fibres lie parallel to the articular surface, and this tangential orientation allows the articular surface to resist shear stresses. In osteoarthritis, this zone is the first to show degenerative changes. Zone 2 is the transitional zone. Here, the fibres are arranged obliquely and the zone forms the transition between the shearing forces of the surface layer and the compressive forces of the deep layer. Zone 3 is the radial or deep zone. This zone accounts for the majority of the cartilage and the perpendicularly arranged collagen fibres distribute loads and resist compression. The layers of articular cartilage and a schematic representation of chondrocyte morphology are shown in **Figures 1.1 and 1.2**.

The chondrocytes in Zones 2 and 3 produce all the components of the ECM. In these zones, the chondrocytes are spherical in shape and although there are no intercellular junctions between chondrocytes, communities of two or more cells form *chondrons* (4). These share the same pericellular matrix, which differs in its composition and has a higher rate of turnover compared to the inter-territorial ECM between the chondrons.

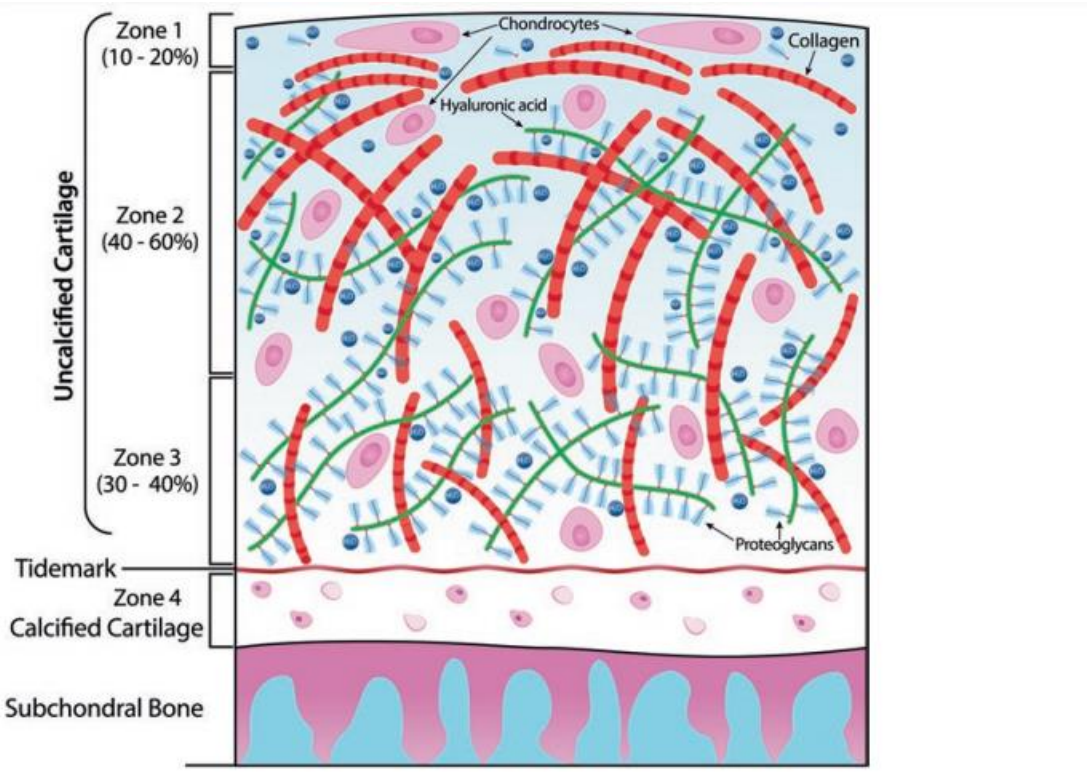

Figure 1.1 Schematic representation of adult knee articular cartilage. The uncalcified cartilage is comprised of three zones (zone 1 to zone 3) which is attached to the subchondral bone via a narrow layer of calcified cartilage (zone 4). The interface between uncalcified and calcified cartilage is demarcated by a thin calcified line called the tidemark.

(Reproduced with permission from: Structure and Function of Articular Cartilage. In: Articular Cartilage of the Knee: Health, Disease and Therapy. Gahunia HK, Gross AE, Pritzker KPH, Babyn PS, Murnaghan L, editors. New York, NY: Springer New York; 2020. p. 3-70).

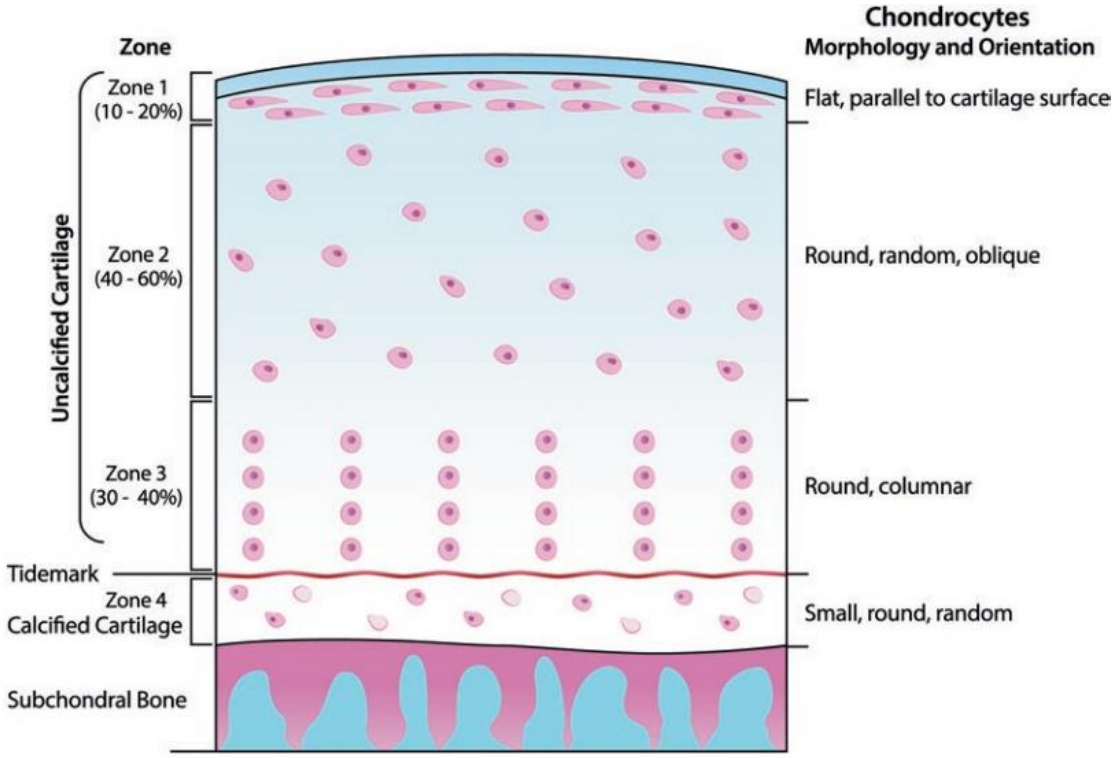

Figure 1.2 Schematic representation of articular cartilage chondrocyte morphology and orientation within the zones. Zone 1 is characterized by small, flat, discoid or ellipsoid chondrocytes with their long axis parallel to the cartilage surface. Zone 2 consists of an obliquely oriented, random distribution of round or oblong chondrocytes with the long axis perpendicular to the cartilage surface. Zone 3 chondrocytes are round and largest in size with columnar distribution, whereas those within the zone of calcified cartilage (zone 4) are smallest in size, round and randomly distributed.

(Reproduced with permission from: Structure and Function of Articular Cartilage. In: Articular Cartilage of the Knee: Health, Disease and Therapy. Gahunia HK, Gross AE, Pritzker KPH, Babyn PS, Murnaghan L, editors. New York, NY: Springer New York; 2020. p. 3-70).

1.1.3 The chondrocyte

The articular chondrocyte is derived from uncommitted mesenchymal stem cells. Chondroblasts proliferate during foetal development and the majority of cartilage transforms into bone through endochondral ossification, with the growth plate and cartilaginous epiphyses persisting after birth. At skeletal maturity, the articular surface is the only remaining cartilaginous part. Articular chondrocytes and growth plate chondrocytes represent two different pathways of terminal differentiation. The matrix and synovial fluid environment play extremely important roles in the maintenance of the phenotype of articular chondrocytes (5).

Being avascular, cell nutrition takes place via diffusion from the synovial fluid. Although the exact mechanism is poorly understood, a simple explanation is that when the joint is not under load, the cartilage (being highly hydrophilic) absorbs synovial fluid. Under load, fluid is squeezed out, and with it metabolic waste products. Absence of joint loading fails to maintain adequate nutrition and has been shown to predispose to degeneration.

The function of the articular chondrocyte is to synthesise and maintain the ECM. This matrix serves to aid in smooth joint articulation and withstand forces that occur when the joint is loaded with weight. The ECM is the predominant constituent of cartilage and the chondrocytes make up only a small amount of total tissue volume (5% wet weight of articular cartilage).

1.1.4 The extracellular matrix

The extracellular matrix is a complex of self-assembled macromolecules. It is composed predominantly of water, fibres (collagen and elastin), proteoglycans, glycoproteins as well as degradative enzymes and extracellular ions (2).

The major constituent of the ECM is water (75% wet weight of articular cartilage) which is held in place by the negative charge of the proteoglycans. This confers cartilage with a high hydrostatic pressure such that when it is compressed, a thin layer of fluid is leaked to the articular surface. This facilitates fluid-film lubrication and reduces friction.

Collagen synthesis takes place in stages both within and outside the chondrocyte. Approximately 90% of the collagen in articular cartilage is Type II (6). These highly cross-

linked fibres of triple helical type II collagen molecules interact with other cartilage-specific collagens such as collagen types IX and XI and give articular cartilage its tensile strength. Type I collagen is not found in normal articular cartilage but is present following injury in the subsequently formed fibrocartilage.

Proteoglycans are responsible for most of the water content of cartilage and also provide compressive strength. They are large hydrophilic molecules containing chains of glycosaminglycans (e.g. chondroitin sulphate and keratin sulphate). Aggrecan is the predominant proteoglycan in articular cartilage.

Glycoproteins such as chondrocalcin, lubricin and laminin are sparsely distributed throughout the ECM. These macromolecules act as 'tissue glue', binding to various constituents of the matrix and the chondrocyte surface.

The homeostasis of cartilage is maintained by complex mechanisms controlling turnover and remodelling of the ECM. Chondrocytes embedded within the ECM produce local factors, inflammatory mediators, and matrix-degrading enzymes (the matrix metalloproteinases) that control the turnover and degradation of normal and pathological ECM.

The ECM is not only a scaffold for the cells; it also serves as a reservoir for growth factors and cytokines and modulates the activation and turnover of the cell (7). The ECM should be considered a dynamic network of molecules, produced by chondrocytes, that in turn regulates cellular behaviour by modulating their proliferation and differentiation.

1.1.5 The role of the chondrocyte in the pathophysiology of osteoarthritis

Chondrocytes respond to physical and biological changes in their environment, i.e. mechanical load (static and dynamic), osmotic pressure, matrix composition and soluble mediators (cytokines, growth factors, reactive oxygen species). In response to an insult, chondrocytes exhibit a transient clonal growth response with increased deoxyribonucleic acid (DNA) synthesis and proliferation, increased proteoglycan and collagen synthesis, increased production of degradative matrix enzyme and chondrocyte death by apoptosis (8, 9). The upregulation of both anabolic and catabolic activity is an attempt to repair and remodel articular cartilage. However, the tipping of this balance to catabolism subsequently leads to degeneration (10).

1.2 Arthrography

Arthrography is an imaging technique which involves the introduction of a contrast agent into the joint space. It can be either direct or indirect. Both methods enhance visualization of the joint space after imaging of the joint is performed. With indirect arthrography, contrast material is injected intra-venously (i.v.) and eventually absorbs into the joint. With direct arthrography, however, contrast material is injected directly into the joint.

1.2.1 Direct arthrography

Direct arthrography is preferred over indirect arthrography as it leads to more homogenous signal intensity throughout the joint and utilises the natural advantages of joint effusion (11). Contrast solution distends the joint capsule, outlines intra-articular structures, and fills tears, leaking through them into the extra-articular space. Direct arthrography is particularly effective in demonstrating rotator cuff tears, osteochondral loose bodies, and subtle abnormalities of the articular cartilage and labrum.

There are several methods to perform direct arthrography (12).

Conventional direct arthrography uses either fluoroscopy or X-Ray to image the joint following the injection of iodinated contrast material. Conventional direct arthrography is especially useful in paediatric patients as it allows visualisation of structures that are not readily identified on standard X-Rays due to their cartilaginous nature. It is the standard of care for assessment of developmental dysplasia of the hip (DDH), allowing detection of soft-tissue impediments as well as evaluation of femoral head sphericity, the reducibility of the femoral head, joint stability and congruency of the hip joint (13). Other uses in paediatric orthopaedics include in Legg-Calvé-Pethes disease, deformity correction (e.g. Blount disease) and joint evaluation in the trauma setting.

Computed Tomography (CT) direct arthrography also uses iodinated contrast material and may be supplemented by air to produce a double contrast CT arthrogram. CT direct arthrography may be used in some patients who have relative contraindications to undergoing magnetic resonance (MR) imaging and has the advantage of shorter examination times. It has been shown to be useful for the evaluation of cartilage, ligament, and labral lesions in the shoulder, elbow, wrist, hip and knee joints (14).

The contrast material used in *MR direct arthrography* contains gadolinium (Gd), which affects the local magnetic field within the joint and appears on the images. MR direct arthrography extends the capabilities of conventional MR imaging. If a joint is filled with gadolinium-containing fluid, the signal in the cavity increases with the use of T1-weighted sequences, and all intra-articular structures, even those with a weak or intermediate signal (including hyaline and fibrous cartilage, ligaments, tendons, joint capsule), are clearly contrasted (15). MR direct arthrography is commonly used in the shoulder joint to evaluate articular cartilage, the biceps-labral complex and the capsulo-ligamentous complex, as well as the elbow, wrist, hip, knee and ankle joints (16).

1.2.2 Direct arthrography technique

Direct arthrography is a two-step procedure involving (i) intra-articular instillation of a contrast solution followed by (ii) imaging of the joint. The arthrogram is usually performed using fluoroscopy and intra-articular needle tip localization is confirmed by injecting a small amount of iodinated contrast agent. Any effusion is aspirated, and a standard volume of contrast solution is administered until the capsule becomes distended (16).

Various combinations of injection solutions (so-called 'injection cocktails') may be utilised. **Table 1.1** provides an overview of common solutions for the hip and shoulder joints. To achieve maximum MR signal intensity, gadolinium formulations are typically diluted to a concentration of 1 mmol/L or 2 mmol/L (15, 16). For conventional and CT direct arthrography, iodinated contrast agents are typically diluted to a concentration of 150 mg/ml (17). Local anaesthetic is used to minimize the pain of capsular distension. The concentration of Iohexol (an iodinated contrast agent) and gadolinium in several common protocols is shown in **Figure 1.1**.

Table 1.1 Injection volumes for MR and CT direct arthrography of the hip and shoulder

	Lidocaine (1%)	Iohexol (300 mg/ml)	Normal Saline	Gadolinium (0.5 mmol/ml)	Solution Volume	Injection Volume
Hip MR Arthrogram						
*Protocol 1	10 ml		10 ml	0.1 ml	20 ml	12-15 ml
†Protocol 2	10 ml	10 ml		0.1 ml	20 ml	8 – 20 ml
‡Protocol 3		2 ml	10 ml	0.1 ml	12 ml	6 – 8 ml
Shoulder CT Arthrogram						
*Protocol 4	10 ml	10 ml			20 ml	12 ml
*Protocol 5	5 ml	10 ml	5 ml		20 ml	12 – 15 ml

* University of Wisconsin Musculoskeletal Radiology. Imaging Protocols. 2021. Available from https://radiology.wisc.edu/

†MRI of the Musculoskeletal System. 5th ed. Berquist T, editor: LWW; 2006.

‡ Chapman & Nakielny's Guide to Radiological Procedures. 7th ed. Watson, N and Jones, H, editors. Elsevier; 2017

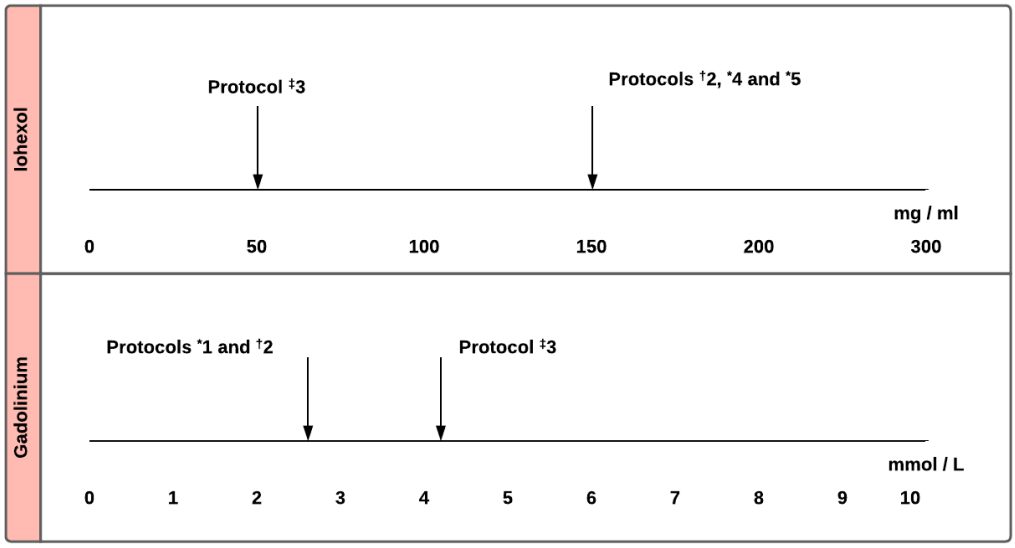

Figure 1.3 Concentration of Iohexol and gadolinium in common MR and CT direct arthrography injection solutions

* University of Wisconsin Musculoskeletal Radiology. Imaging Protocols. 2021. Available from https://radiology.wisc.edu/

†MRI of the Musculoskeletal System. 5th ed. Berquist T, editor: LWW; 2006.

‡ Chapman & Nakielny's Guide to Radiological Procedures. 7th ed. Watson, N and Jones, H, editors. Elsevier; 2017

1.3 Clinical pharmacology of contrast agents

1.3.1 Physical and chemical properties of contrast agents

1.3.1.1 Physical and chemical properties of iodinated contrast agents

Iodinated contrast agents are chemically modified from a 2, 4, 6-triiodinated benzene ring. They are classified according to their chemical structure, iodine content, osmolarity and ionization solution (18). Benzene is a toxic hydrophobic molecule and iodinated contrast agents are usually monomeric (one benzene ring) or dimeric (two benzene rings combined). Iodine has key properties that are essential for contrast medium: (i) high-contrast density, (ii) high stability for the benzene binding and (iii) low toxicity (19). Introducing side-chains in positions 3 and 5 renders triiodobenzoic acid even less toxic and more water-soluble. Ionic

agents are able to break up into a cation and anion and have high osmolality. Non-ionic agents do not have this ability and are therefore less osmolar. Ionic agents are classified as high-osmolar (HOCM, 2000 mOsm/kg; 5 to 8 times the osmolarity of plasma) or low-osmolar (LOCM, 600-800 mOsm/kg; 2 to 3 times the osmolarity of plasma). Non-ionic agents are classified as low-osmolar or iso-osmolar (IOCM, 290 mOsm/kg; same osmolarity as blood, plasma and cerebrospinal fluids). HOCM are older contrast agents and considered more nephrotoxic than newer LOCM and the increasingly used IOCM agents (19, 20).

1.3.1.2 Physical and chemical properties of gadolinium contrast agents

Gadolinium is a member of the lanthanide group of elements, which all possess partially filled inner shells of electrons. Electrons have a large charge-to-mass ratio and exert strong magnetic moments. In its native state, Gd is paramagnetic and has seven unpaired electrons in its 4f orbital, more than any other element (19). Free Gd is extremely toxic, however; it is an inorganic blocker of voltage-gated calcium channels, inhibiting physiological processes that depend on Ca^{2+} influx as well as the activity of some metabolic enzymes (21). It has also been shown to increase the expression of hepatic cytokines and can cause severe hepatic necrosis (20). Therefore, all Gd-contrast agents are chelates that contain the Gd ion (Gd^{3+}). There is increasing evidence that chemical stability of the chelate has a great influence on the exchange between Gd^{3+} and other body metal ions (notably Zn^{2+}, Cu^{2+} or Ca^{2+}), a process known as transmetallation, which leads to increased accumulation of free Gd^{3+} in bones and skin (22). There are two structurally distinct categories of Gd chelates, namely linear and cyclic chelates. Cyclic molecules provide better protection and binding to Gd^{3+} compared to linear molecules (23). Thermodynamic stability and kinetic inertness further influence Gd^{3+} stability and cyclic chelates with high thermal stability constants and long dissociation half-lives are least likely to release free Gd^{3+} (24, 25).

1.3.2 Total body dose of contrast agents following intra-articular administration

Gd-based contrast agents are licensed for i.v. administrations up to a total body dose of 0.3 mmol/kg in adults. The standard dose however, is 0.1 mmol/kg body weight. Given a concentration of 2 mmol/L and a patient weight of 70 kg, a theoretical total body dose of 50 ml x 2µmol/ml divided by 70 kg = 1.4 µmol/kg results from a maximum administered volume of 50 ml. Depending on the size of the joint, a volume of 10 to 40 ml is usually injected in direct arthrography. Therefore, the theoretical maximum whole body dose after

intra-articular administration is approximately 200 times lower than the maximum permissible and approved intra-venous dose (15).

Similarly, the recommended i.v. dose of Iohexol (300 mg/ml) is 2.0 ml/kg in adults, with the total i.v. administration dosage not to exceed 3.0 ml/kg (26). With a patient weight of 70 kg, an intra-articular injection of 10 ml of Iohexol (300 mg/ml) results in a theoretical total body dose of 0.14 ml/kg, 21 times lower than the maximum permissible and approved intra-venous dose.

1.3.3 Elimination of intra-articular contrast agents

Only a few clinical studies exist which permit at least an estimation of the timeframe in which intra-articular contrast agents leave the joint. According to a study which examined the imaging window after an injection of 2 mmol/l Gd-DTPA solution into the shoulder joint, the highest signal intensity was achieved immediately after injection, and fell within the first 120 minutes. The level of usable contrast in the joint was non-diagnostic after 6 and 24 hours (27, 28). Similarly, Kokkonen et al. investigated the feasibility of delayed CT arthrography of the knee joint (29). Two hours after injection, the concentration of intra-articular contrast agent was still high enough (>20% of the initial concentration at 0 minutes) to allow delayed CT arthrography. The half-life of the contrast agent in the joint averaged 45 minutes and concentration in patellar and femoral cartilage reached its maximum value after 30 and 60 minutes, respectively.

Just as after i.v. administration, it appears that complete and rapid renal elimination takes places following intra-articular injection and passive diffusion out of the joint. No significant amount of contrast agents have been demonstrated in recovered tissue, either in animal experiments or pathological studies. Engel et al. (30) showed that while free gadolinium is taken up completely by hyaline cartilage (i.e. by all three zones) within 24 hours, complex-bound Gd-DTPA diffuses much more slowly and in much smaller amounts into cartilage tissue. These results were subsequently confirmed in 10 patients undergoing total knee replacement who were administered a Gd-DTPA solution (1 – 5 mmol/L) at a median pre-operative time of 31.5 hours (range, 17 to 137 hours). Resected cartilage specimens were examined by mass spectroscopy for their Gd content and almost all values were near the lowest limit of detection (0.05 ppm).

1.4 Toxicity of contrast agents

During the last few decades, there has been a marked increase in the use of medical imaging, with a corresponding rise in the use of iodinated and Gd-based contrast agents. Over half of the approximately 76 million CT and 34 million MR imaging exams performed each year involve the use of contrast media (31, 32). Although routinely used in clinical practice both classes of agents possess adverse side-effects.

1.4.1 Toxicity of iodinated contrast agents

While iodinated contrast agents are generally considered to be safe, there are well-known risks in patients with impaired kidney function, partly owing to their decreased renal excretion (33). In addition, contrast agents may lead to drug-drug interactions with patients' pre-existing medications (34). The incidence and severity of these adverse effects has been shown to be related to the osmolality of the agent (19). Adverse effects of iodinated contrast agents may be divided into renal and general side-effects.

1.4.1.1 Renal adverse effects of iodinated contrast agents

Contrast-induced nephropathy (CIN) is a potentially life-threatening acute decline in renal function following administration of iodinated contrast media. Patients with chronic kidney disease (CKD, Stage 3 to 5) – approximately 19 million adults in the U.S. – are the principal at-risk group (19). CIN accounts for over 10% of annual cases of hospital-acquired renal insufficiency and carries a significant morbidity and mortality (35, 36).

On a basic level, acute kidney injury is caused by direct toxicity to the tubular cells (including apoptosis) as well as renal medullary ischaemia. HOCM have been shown induce significantly greater apoptosis in human renal tubular epithelial cells compared to LOCM (37). Other toxic effects include cellular energy failure, disruption of calcium homeostasis and disturbance of tubular cell polarity, with non-ionic LOCM having less toxicity (38). The molecular mechanisms of the direct toxicity remain unclear, although oxidative stress has been implicated (19).

1.4.1.2 General adverse effects of iodinated contrast agents

Besides nephropathy, other adverse reactions may occur after administration of iodinated contrast agents. These may be acute (within 1 hour of administration) or delayed (after 1 hour but within 1 week). Acute reactions are not dose-related and are labelled 'anaphylactoid' as

they have the features of anaphylaxis, but are IgE negative (39). Mild acute adverse reactions include flushing, pruritus and nausea; moderate acute adverse reactions include hypotension and bronchospasm; severe acute adverse reactions include convulsion, laryngeal oedema, pulmonary collapse and cardiac arrest (19). Delayed adverse reactions include headache, parotitis and flu-like illness. Anaphylactoid reactions are more common with HOCM, whereas cardiovascular events are more common with LOCM (40, 41).

1.4.2 Toxicity of gadolinium-based contrast agents

1.4.2.1 Nephrogenic systemic fibrosis

Nephrogenic systemic fibrosis (NSF) is a serious, potentially fatal condition that occurs in patients following administration of Gd-based contrast agents who have severe chronic or acute renal failure. It is characterized by the formation of connective tissue in the skin but can also affect other organs including the lungs, pleura, skeletal muscle, heart, pericardium and kidneys (42).Typical findings include rapidly progressive thickening of the skin, tethering and hyperpigmentation mainly involving the extremities, progressing cephalad from the legs and feet (43). NSF develops over a period of days to several weeks and approximately 5% of patients have a rapidly progressive severe disease course (19). However, some studies have shown onset as long as eight years after exposure (44). The association between NSF and Gd-based contrast agents was discovered in 2006 (45) but the relationship remains incompletely understood. Some investigators speculate that it is related to the dissociation of free gadolinium from the chelate, which binds to anions. This may produce an insoluble precipitate that is subsequently deposited in tissues, inciting a fibrotic reaction (46). Non-ionic linear Gd-chelates are associated with the highest risk of NSF (19).

1.4.2.2 General adverse effects of gadolinium-based contrast agents

The incidence of general adverse effects with Gd-based contrast agents is much lower than that of iodinated contrast agents. The most frequently observed adverse reactions include transient headache, nausea and emesis. Allergic reactions have also been reported (19).

1.5 Morbidity of direct arthrography

Detailed examination of the local and general tolerance of intra-articular contrast agents have been made in large open-label prospective studies. Although well-established and largely considered to be safe, direct arthrography is an invasive procedure with an associated patient morbidity that may be underestimated (47). Post-arthrographic pain is commonplace, yet the cause of this pain remains to be fully elucidated.

1.5.1 Post-arthrographic pain

Heinz et al. used "patient cards" to assess the tolerance of direct arthrography in 1083 patients (48). A "feeling of pressure in the joint" was experienced by 67% of patients on the day of the examination, by 29.5% on the second day and by 15% on the third day. "Pain on moving" was reported by 61.6% of patients on the day of the examination, by 49% on the second day and by 38% on the third day. A multi-centre trial of 470 direct arthrography patients found similar results with a continuous, significant decrease in patients' complaints during the first two post-procedure days (49). Giaconi et al. noted post-arthrographic pain in 66% of patients undergoing direct arthrography which lasted an average of 44.4 ± 30.5 hours (47). On an 11-point numeric pain rating scale, the average pain intensity was 4.8 (range, 1 to 10) with an average time to onset of pain after joint injection of 16.6 hours (range, 4 to 72 hours). Hall et al. reported a similar incidence of pain following conventional shoulder arthrography of 74% (50).

Early reports focused on joint distension as the cause of post-arthrographic pain (15). However, direct irritation of the synovium resulting in chemical synovitis may play a role and synovial hyperaemia has been noted at the time of arthrotomy performed within a few days of direct arthrography (50). The time course of pain after contrast medium injection is compatible with chemical synovitis and (51) an inflammatory response may explain why all patients do not develop post-procedure pain. Relief of post-arthrographic pain by non-steroidal anti-inflammatory drugs and ice lends further support to this theory. Post-arthrographic pain has also been related to the influx of fluid, which in turn depends on the osmolarity of the contrast agents. However, this was not supported by Andresiek et al. who found that the volume of contrast media decreased rather than increased within 45 to 90 minutes following injection in the shoulder, hip and wrist (52).

1.5.2 Systemic safety of direct arthrography

For systemic safety, and therefore the possible influence on laboratory parameters, the total amount of contrast agent applied is more important than the local intra-articular concentration. No extensive studies exist regarding the effect of intra-articular administration of contrast agents on clinico-chemical parameters, particularly in patients with impaired kidney or liver functions (15). However, because of the identical elimination of contrast agents after intra-venous and intra-articular administration, the extensive experience with i.v. administration is valid. Moreover, the maximum whole body dose after intra-articular administration of both iodinated and Gd-based contrast agents are much lower than the maximum permissible and approved i.v. doses of each (Section 1.3.2).

1.6 Chondrotoxicity of local anaesthetics

Chondrolysis is the irreversible destruction of previously healthy articular cartilage resulting from the loss of chondrocytes that maintain the ECM (53, 54). An inciting event produces early cartilage deterioration, which in turn, triggers an inflammatory cascade within the joint. Once initiated, diffuse chondrocyte death occurs over a short period of time, leading to extensive cartilage loss with devastating consequences. Pain and disuse changes further complicate limb dysfunction and ultimately, many patients require some form of joint arthroplasty (55).

As chondrolysis cannot be reversed, its occurrence can only be prevented by establishing and avoiding its causes (56). There are many published reports of post-arthroscopic glenohumeral chondrolysis (PAGCL) following the intra-articular infusion of local anaesthetic via a pain pump in the shoulder. These reports initiated a cascade of *in vitro* and *in vivo* studies that undoubtedly confirmed the toxicity of local anaesthetics to human and animal articular cartilage.

1.6.1 Clinical reports

To our knowledge, the first documented case of chondrolysis following post-operative intra-articular infusion of local anaesthetic via a pain pump was made by Petty et al. in 2004 (57). In this instance, an 18 year old female college athlete underwent routine shoulder arthroscopy with subacromial decompression and rotator cuff debridement for impingement. Intra-operatively, she was noted to have healthy-appearing cartilage and at the procedure end, a

pain pump was inserted with 0.5% bupivacaine and epinephrine (adrenaline). Due to ongoing pain and limited movement, however, a second arthroscopy was performed six months later. Severe glenohumeral arthritis was observed with complete loss of the glenoid cartilage and extensive loss of the humeral head cartilage. There was no osteophyte formation and no evidence of infection.

Since then, the reported incidence of glenohumeral chondrolysis has risen rapidly. In 2010, a systematic review of 100 previously published cases revealed that 59% were in shoulders that had received local anaesthetic infusion via a pain pump catheter (58). Two years later, the total number of reported shoulders with glenohumeral chondrolysis had more than doubled to 213 (56). Of the 113 new cases, 110 (97%) were in shoulders that had received intra-articular local anaesthetic via a pain pump. The typical findings were of normal-appearing cartilage at the index procedure, followed in a few months by the onset of pain and stiffness associated with global cartilage loss (56).

Chondrolysis following intra-articular infusion of local anaesthetic has also been documented in other joints. Noyes et al. reported severe chondrolysis in 21 knees following the use of either a high or low-flow-volume pump infusing intra-articular bupivacaine (55). The index procedures included 18 anterior cruciate ligament (ACL) reconstructions, 1 meniscal repair, 1 arthroscopy and 1 tibial tubercle osteotomy. Chondrolysis lead to disabling knee symptoms in all patients, which failed to respond to a variety of interventions. Meanwhile, Bojescul et al. reported the case of a 21 year old West Point cadet who underwent routine arthroscopy and ankle stabilisation with a modified Brostrom procedure (59). Post-operative analgesia involved a bupivacaine continuous infusion pump system. The patient returned to full activities after 4 months but had re-presented within the year with increasing pain and stiffness. Second-look arthroscopy revealed diffuse chondrolysis of the ankle joint.

1.6.2 In vitro studies

A number of studies have utilised *in vitro* models to assess the time- and dose-dependent effects of local anaesthetic agents on chondrocyte cultures (**Table 1.2**).

1.6.2.1 Lidocaine

Farkas et al. (60) exposed chondrocyte monolayers and osteochondral explants obtained from the unaffected lateral femoral condyles of patients undergoing total knee arthroplasty (TKA) to various local anaesthetic agents. They found significant differences in the number of apoptotic cells when comparing lidocaine 1% to phosphate-buffered saline (PBS) at 6 hours and 24 hours of exposure.

Similarly, Grishko et al. (61) generated primary chondrocyte cultures from cartilage specimens obtained during TKA and measured cell viability and apoptosis at 24 and 120 hours after a 1-hour treatment. They found evidence of dose-dependent toxicity with lidocaine 2% leading to almost complete less of viable cells at 24 hours due to massive necrosis. In contrast, lidocaine 1% caused a detectable, but not significant decrease in viability and lidocaine 0.5% did not affect chondrocyte viability at 24 hours. However, both lidocaine 1% and 0.5% had significantly lower viability at 120 hours compared to a saline control.

Jacobs et al. (62) suspended human articular chondrocytes in alginate beads and exposed them to lidocaine 1% and 2% for various time periods. After 1 hour exposure, devastating results were seen for lidocaine 1% and 2% with cell death rates of 91% and 99%, respectively, compared to 26% in a saline control. Moreover, lidocaine demonstrated a time-dependent toxicity with gradually more dead cells at 7 days following exposure for 15, 30 or 60 minutes.

Using a bioreactor with a continuous infusion pump to mimic joint fluid metabolism, Braun et al. (63) assessed the effects of a single injection of local anaesthetic on human, non-transduced, unmodified chondrocytes from a commercially available cell line. They found a significant decline in cell viability on day 14 following injection of lidocaine 1% (9.8%) compared to a media control (5.5%).

Dragoo et al. (64) also used a custom bioreactor to assess various local anaesthetic agents over their clinical duration of action on chondrocyte monolayers as well as intact cartilage samples. They found that human chondrocytes treated for 3 hours with a single dose of lidocaine 1% exhibited significantly more cell death (7.9%) compared to a media control (2.9%).

Rao et al. (65) expanded commercially available human articular chondrocytes into monolayer cultures and exposed them to a combination of local anaesthetics for various time periods. Cell viability was then assessed at 1, 3, 5 and 7 days after exposure. At all time points, lidocaine 1% resulted in a significant increase in chondrocyte apoptosis compared to a saline control.

1.6.2.2 Bupivacaine

Chu et al. (66) obtained human articular cartilage from tissue donors and TKA patients as well as bovine articular cartilage from animals within four hours of their slaughter. Chondrocytes were isolated and re-suspended within alginate bead cultures before being immersed in varying concentrations of local anaesthetic for 15, 30 or 60 minutes. Overall, they found a significant time-dependent and dose-dependent chondrotoxic effect when comparing both bupivacaine 0.5% and bupivacaine 0.25% to a saline control. However, exposure to bupivacaine 0.125% showed no difference in cell viability when compared to a saline control at any time point.

Piper et al. (67) harvested macroscopically normal articular cartilage from five patients undergoing either TKA or bipolar hemiarthroplasty of the hip. Chondrocyte monolayers and cartilage explants were exposed to different local anaesthetics and cell viability was assessed at 24 hours. With regard to bupivacaine 0.5%, chondrocyte viability in both the monolayer cultures and cartilage explants were significantly decreased compared to either ropivacaine or a saline control.

Farkas et al. (60) found significant differences in the number of apoptotic cells when comparing bupivacaine 0.5% to PBS at 2, 6 and 24 hours of exposure. Moreover, they found that bupivacaine 0.5% was more chondrotoxic than either lidocaine 1% or ropivacaine 0.75% as it induced almost 100% cell necrosis after 24 hours of exposure.

Grishko et al. (61) found that while bupivacaine 0.25% did not affect cell viability at the time points analysed up to 120 hours, bupivacaine 0.5% caused a significant decline at both 72 and 120 hours.

Baker et al. (68) used commercially available normal human chondrocytes to produce monolayer cultures that were then exposed to different local anaesthetics across a range of concentrations. With regard to bupivacaine, they found a significant dose-dependent reduction in cell viability when compared to saline at each concentration tested.

Syed et al. (69) obtained cartilage from the femoral heads of three patients undergoing bipolar hemiarthroplasty and set up chondrocyte monolayers that were exposed to bupivacaine 0.25% either alone or in combination with steroid. 1 hour after a 15-minute exposure, they found evidence of bupivacaine 0.25% toxicity (71.8% viability relative to a saline control).

In contrast, Dragoo et al. (64) found no significant increase in cell death in human chondrocytes treated for 6 hours with a single dose of bupivacaine 0.25% (2.7%) compared to a media control (2.9%).

Breu et al. (70) harvested articular cartilage from the femoral heads of four patients undergoing total hip arthroplasty (THA). While exposure to bupivacaine concentrations up to 0.25% did not reveal significant chondrotoxicity in flow cytometry, they found that bupivacaine 0.5% caused a significant increase in apoptotic and necrotic cells when compared to buffer saline controls. In addition, they found that bupivacaine 0.5% was more chondrotoxic than ropivacaine 0.75%.

Cobo-Molinos et al. (71) used cartilage from five patients undergoing either THA or TKA to set up chondrocyte monolayer and articular cartilage explant cultures. They found that exposure to bupivacaine 0.5% for 15 or 30 minutes did not significantly affect chondrocyte viability. However, after 1 hour of exposure, chondrocyte mortality in the cartilage explants (20.7%) was significantly greater with a saline control (9.6%).

Ickert et al. (72) suspended chondrocytes in alginate beads and set up high dose/long duration and low dose/short duration experiments to compare the effects of local anaesthetic and opioid agents against saline controls. Cartilage was harvested from nine patients undergoing either total joint arthroplasty or amputation of polydactylous toes. They found dose- and time-dependent chondrotoxic effects for bupivacaine and furthermore, bupivacaine 0.5% appeared more chondrotoxic that ropivacaine 0.75%.

When considered across all time points, Rao et al. (65) found that bupivacaine 0.5% resulted in significantly greater chondrocyte death than ropivacaine 0.5%. Chondrocyte death was also greater compared to lidocaine 1%, but this did not reach statistical significance. At 1, 3, 5 and 7 days following exposure, bupivacaine 0.5% resulted in a significantly increased rate of chondrocyte apoptosis compared to a saline controls.

1.6.2.3 Ropivacaine

In cartilage explants exposed to either saline or ropivacaine 0.5% for 30 minutes, Piper et al. (67) did not find a significant difference, with predominantly live cells being present at 24 hours in both sets of implants from the articular surface throughout their depths (1mm). Nonetheless, ropivacaine 0.5% did lead to a significant decline in cell viability in monolayer cultures, but the authors noted that ropivacaine 0.5% was less chondrotoxic than bupivacaine 0.5%.

Baker et al. (68) found that ropivacaine lead to a dose-dependent reduction in cell viability in monolayer cultures while Farkas et al. (60) showed that ropivacaine 0.75% resulted in a time-dependent decrease in chondrocyte viability when compared to a PBS control. Similar to Piper et al. (67), ropivacaine was found to be less chondrotoxic than bupivacaine.

Although Grishko et al. (61) did not directly compare the three local anaesthetic agents in their study, lidocaine 1% and bupivacaine 0.5% appeared to result in a greater decrease in cell viability when compared to ropivacaine 0.5% at 24, 72 and 120 hours.

Evaluating the effect of a single dose of various local anaesthetic agents, Dragoo et al. (64) did not find a significant difference in chondrocyte death between cells treated for 12 hours in ropivacaine 0.5% (2.9%) versus a media control (2.5%).

In the study by Breu et al. (70), 60-minute exposure to various ropivacaine concentrations from 0.031% to 0.75% did not significantly increase the number of apoptotic or necrotic cells at 24 hours, but ropivacaine 0.75% did result in a significant increase in necrosis at 96 hours. Chondrotoxic effects were greater for bupivacaine than ropivacaine

Finally, both Ickert et al. (72) and Rao et al. (65) noted chondrotoxic effects for ropivacaine at exposure times ≥ 30 minutes but again, each study found that bupivacaine was more chondrotoxic than ropivacaine.

Table 1.2 *In vitro* studies of the chondrotoxic effect of local anaesthetics on human articular cartilage

Author	Year	Local Anaesthetic	Model	Treatment Protocol	Outcome Measurement
Chu et al. (66)	2008	Bupivacaine	Human chondrocytes	Bupivacaine 0.125%, 0.25%, 0.5% 15, 30, 60 mins.	Flow cytometry
Piper et al. (67)	2008	Bupivacaine/ Ropivacaine	Human cartilage explant	Bupivacaine 0.5%; Ropivacaine 0.5% 30 mins.	Live/Dead Viability/Cytotoxicity assay
			Human chondrocytes		CellTiter-Glo Luminescent Cell Viability Assay
Farkas et al. (60)	2010	Lidocaine/ Bupivacaine/ Ropivacaine	Human cartilage explant	Lidocaine 1%; Bupivacaine 0.5%; Ropivacaine 0.75% alone and in combination with steroids	TUNEL assay
			Human chondrocytes	2, 6, 24 hours	Flow Cytometry
Grishko et al. (61)	2010	Lidocaine/ Bupivacaine/ Ropivacaine	Human chondrocytes	Lidocaine 0.5%, 1%, 2%; Bupivacaine 0.25%, 0.5%; Ropivacaine 0.2%, 0.5% 1 hour	Flow cytometry/ Nuclear staining and caspase 3 and 9 cleavage assays (Western blot)
Baker et al. (68)	2011	Levobupivacaine/ Bupivacaine/ Ropivacaine	Human chondrocytes	Levobupivacaine 0.13%, 0.25%, 0.5%; Bupivacaine 0.13%, 0.25%, 0.5%; Ropivacaine 0.19%, 0.38%, 0.75% 15 mins.	CellTiter 96® Aqueous One Solution Cell Proliferation Assay

Jacobs et al. (62)	2011	Lidocaine	Human chondrocytes	Lidocaine 1%, 2% 15, 30, 60 mins.	LDH activity/ Il-6 production/ Live/Dead cell count
Syed et al. (69)	2011	Bupivacaine	Human cartilage explant	Bupivacaine 0.25% alone and in combination with triamcinolone	Live/Dead Viability/Cytotoxicity assay
			Human chondrocytes	15 mins.	MTT assay
Braun et al. (63)	2012	Lidocaine/ Bupivacaine	Human chondrocytes	Lidocaine 1%; Bupivacaine 0.25% alone and in combination with various corticosteroids Bioreactor and continuous infusion pump 7, 9 or 14 days	Live/Dead Viability/Cytotoxicity assay
Dragoo et al. (64)	2012	Lidocaine/ Bupivacaine/ Ropivacaine	Human cartilage explant	Lidocaine 1%; Bupivacaine 0.25%; Ropivacaine 0.5%	ECM damage evaluated by analysis of DNA, GAG and collagen content
			Human chondrocytes	Bioreactor and continuous infusion pump 3, 6 or 12 hours	Live/Dead Viability/Cytotoxicity assay
Breu et al. (70)	2013	Bupivacaine/ Ropivacaine	Human cartilage explant	Bupivacaine 0.25%, 0.5%; Ropivacaine 0.5%. 0.75%	Fluorescence microscopy
			Human chondrocytes	1 hour	Flow cytometry/ Live-Dead staining/ Caspase detection

Cobo-Molinos et al. (71)	2014	Levobupivacaine/ Bupivacaine	Human chondrocytes	Levobupivacaine 0.5%; Bupivacaine 0.5% 15, 30, 60 mins.	Flow cytometry/ Live-Dead staining
Ickert et al. (72)	2014	Bupivacaine/ Ropivacaine	Human chondrocytes	Bupivacaine 0.5%, Ropivacaine 0.75% 15, 60, 240 mins.	CellTiter-Glo® luminescent cell viability assay for adenosine triphosphate
Rao et al. (65)	2014	Lidocaine/ Bupivacaine/ Ropivacaine	Human chondrocytes	Lidocaine 1%; Bupivacaine 0.5%; Ropivacaine 0.5% 30 to 120 mins. in 15 mins. divisions	Live/Dead Viability/Cytotoxicity assay/ Caspase-3 staining/ TUNEL assay

TUNEL, Tdt-mediated dUTP neck-end labelling; LDH, Lactate Dehydrogenase; Il-6, Interleukin-6; MTT, [3-(4, 5-dimethylthiazol-2-yl)-2, 5-dephenyl tetrazolium bromide]; ECM, Extracellular Matrix; DNA, Deoxyribonucleic Acid; GAG, Glycosaminoglycan;

1.6.3 In vivo studies

A series of *in vivo* studies have corroborated the *in vitro* evidence that local anaesthetics are toxic to articular chondrocytes.

Gomoll et al. (73) reported profound chondrotoxicity for local anaesthetics in an experimental model that mimicked the clinical application of post-operative pain pumps. Rabbits were divided into three groups to receive 48-hour continuous intra-articular shoulder infusions of either saline, bupivacaine 0.25% or bupivacaine 0.25% with adrenaline (1:200,000). The animals were sacrificed one week later and osteochondral samples were analysed with confocal microscopy and histological analysis. In comparison with the saline control, treatment with bupivacaine 0.25% both with and without adrenaline caused significant histopathological and metabolic changes and reduced cell viability by 20% and 32%, respectively.

Other studies have focused on the potential effects of a single intra-articular injection of local anaesthetic agent.

In a rabbit model Dogan et al. (74) investigated the short-term effect of a single intra-articular injection of local anaesthetic on articular cartilage and synovial membrane. Knees were injected with either bupivacaine 0.5% or a saline control and histological analysis performed 1, 2 or 10 days later. At each time point, joints treated with bupivacaine 0.5% demonstrated significantly greater inflammation of the articular cartilage compared with a saline control.

Chu et al. (75) examined the long-term effects of a single intra-articular injection of local anaesthetic on the articular cartilage of Sprague-Dawley rats. Using three-dimensional confocal reconstructions of fluorescent-stained tissues throughout the distal part of the femur, they were able to show a reduction in chondrocyte density without cartilage loss six months after a single injection of bupivacaine 0.5%, suggesting chondrotoxicity. However, the chondral surfaces remained intact on gross and histopathological examination with no difference in superficial chondrocyte viability between the saline and bupivacaine-treated knees at any time point. The authors concluded that the *in vivo* effects of a single intra-articular injection of bupivacaine 0.5% are likely subtle and any potential toxic effects would be difficult to detect clinically.

More recently, Iwasaki et al. (76) examined the effect of multiple local anaesthetic injections in a rat model. Sprague-Dawley rats received an intra-articular knee injection of either bupivacaine 0.5% or saline solution once a week for five consecutive weeks. The rats were sacrificed at 8, 16 and 24 weeks and osteochondral samples were assessed with confocal microscopy and histological analysis. No significant differences in cell viability, cell density or histological assessment were found between the saline solution-treated and bupivacaine-treated groups at any time point.

To the best of our knowledge, only one human study has reported the effect of a single intra-articular injection of local anaesthetic. Ravnihar et al. (77) performed a retrospective analysis of 49 consecutive patients assigned for autologous chondrocyte implantation (ACI). Cartilage biopsy had been performed either under general/spinal anaesthesia or local anaesthesia (with a single intra-articular injection of 15 – 20 ml of lidocaine 2%). Interrogation of patient data from their national ACI registry demonstrated no inter-group differences in either chondrocyte viability, morphology or cultivation potential. Limitations of this study, however, include the short exposure time to local anaesthetic prior to introduction of the arthroscope with fluid inflow (< 20 minutes) and the lack of a comparative, standardised control group.

1.7 Chondrotoxicity of contrast agents

Despite their widespread use in musculoskeletal imaging, few studies have examined the effects of contrast agents on articular cartilage. Clinical reports have focused on acute toxicity and adverse reactions (Section 1.4) but with regard to local effects after intra-articular administration, limited data is available.

1.7.1 In vitro studies

Rahmouni et al. (78) examined chondrocyte viability and cell proliferation following treatment with two concentrations of Gd-DOTA (gadolinium tetra-azacyclododecane tetraacetic acid). In one experiment, a monolayer of rabbit chondrocytes was treated with either Gd-DOTA 5 mmol/L, Gd-DOTA 50 mmol/L or a media control for 90 minutes. Cell viability was then estimated by their ability to take up neutral red. In a second experiment, cell proliferation was assessed following incubation for 24 or 48 hours with either 5 mmol/L or 50 mmol/L Gd-DOTA added to the culture media. The volume of Gd-DOTA added was not provided. No cytotoxicity was noted at a concentration of 5 mmol/L but at a concentration of 50 mmol/L, chondrocyte proliferation was markedly reduced at both 24 and 48 hours when compared with a media control.

Greisberg et al. (79) treated bovine chondrocyte monolayers with varying concentrations of gadodiamide (4, 20, 40 and 200 mmol/L) for 16 hours and noted a dose-dependent decrease in cell proliferation and increase in apoptosis.

Midura et al. (80) used a long-term Swarm rat chondrosarcoma chondrocyte-like cell line to examine the effects of 48 hours exposure to varying concentrations of gadopentetate dimeglumine (Gd-DTPA). Cell proliferation of cultures treated with either 1 mmol/L or 5 mmol/L Gd-DTPA did not appreciably differ from untreated controls, but a concentration of 7.5 mmol/L lead to a 22% reduction in DNA content that approached statistical significance. At all doses, spectrophotometric assay of metabolic activity found no difference from untreated controls.

To the best of our knowledge, only one study has examined the potential chondrotoxic effects of contrast agents using human articular chondrocytes. This is also the only *in vitro* study to investigate possible cytotoxic effects of an iodinated contrast agent on chondrocytes. Oznam et al. (81) generated chondrocyte monolayers from the cartilage specimens of six patients undergoing TKA. Cultures were divided into four groups before treatment with various

agents for 2 or 6 hours. Group 1 had no drug exposure, serving as the control group; Group 2 were treated with iopromide 75 mg/ml; Group 3 were treated with Gd-DPTA 20 mmol/L; and Group 4 were treated with a mixed contrast solution containing lidocaine (achieving final concentrations of iopromide 75 mg/ml and Gd-DTPA 2 mmol/L). Compared with the control group, cultures treated with Gd-DTPA 20 mmol/L (Group 3) had the lowest chondrocytic activity and lowest cell viability at six hours. A significant decrease in cell viability was also noted at six hours with the mixed solution (Group 4) and the authors advised against the use of local anaesthetic in direct arthrogram solutions.

1.7.2 In vivo studies

Three *in vivo* studies have reported the effects of intra-articular contrast agents on animal cartilage.

Hajek et al. (82) described the gross pathologic and histologic alterations induced in the synovial membrane of rabbit knees following intra-articular administration of Gd-DTPA 0.5 mmol/L. Animals were sacrificed at 2, 6, 12 or 24 hours or 8 days after the injection, and while the study's primary focus was on synovial changes, the authors documented macroscopically normal appearing cartilage. X-Ray fluorescent spectroscopy revealed no evidence of Gd-DTPA in the synovium or articular cartilage at any time point (with a lower border of detectability for Gd-DTPA of 5-10 µmol/L).

Rahmouni et al. (78) injected the stifle joint of beagle dogs with Gd-DOTA 50 mmol/L and performed post-mortem examination at 2, 24 or 96 hours after the injection. No evidence of macroscopic or microscopic change was found in the articular cartilage.

Lastly, Kose et al. (83) examined the short-term effect of high-dose contrast agents on articular cartilage and synovium in a rabbit model. Knees were injected with either Gd-DTPA, iopromide or a saline control and histological analysis performed 1 hour, 1 day, 1 week or 2 weeks later. The actual concentration of each contrast agent was not provided. Although the cartilage appeared grossly normal at each time point, histological and ultrastructural alterations were observed, including chondrocyte hypertrophy and an increase in dense glycogen accumulation, large lipid vacuoles and matrix material. The adverse effects of the contrast agents gradually reduced by the second week but did not completely resolve within the study period.

1.8 Objectives

This research will detail the effects of four routinely used intra-articular contrast agents on primary human chondrocyte cell viability. The additive effect of local anaesthetic, which is often included in direct arthrogram solutions, will also be examined. At the time of submission, this is only the second study to report the effect of iopromide and gadopentetate dimeglumine on primary human articular chondrocytes and the first to evaluate the effect of iohexol and gadobenate dimeglumine.

In summary, the principle objectives of this research are as follows:

1. To determine the time-dependent and dose-dependent effect of Iohexol on primary human articular chondrocyte cell viability.
2. To determine the time-dependent and dose-dependent effect of Iopromide on primary human articular chondrocyte cell viability.
3. To determine the time-dependent and dose-dependent effect of Gadopentetate Dimeglumine on primary human articular chondrocyte cell viability.
4. To determine the time-dependent and dose-dependent effect of Gadobenate Dimeglumine on primary human articular chondrocyte cell viability.
5. To determine the time-dependent and dose-dependent additive effect of lidocaine local anaesthetic on primary human articular chondrocyte cell viability when included in arthrogram injection solutions.

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Chapter 2

Materials and Methods

2.1 Ethical Approval

Non-arthritic human articular cartilage was obtained from the femoral heads of patients with displaced intra-articular femoral neck fractures who underwent bipolar hemiarthroplasty at the Mater Misericordiae University Hospital, Dublin, Ireland. The study conformed to the ethical guidelines of the 1975 Declaration of Helsinki, as reflected in a priori approval by the Local Ethical Committee of the Mater Misericordiae University Hospital. All patients were provided with a detailed information leaflet pre-operatively and consented to retrieval of their femoral head.

2.2 Materials

2.2.1 Femoral Heads

Femoral heads were obtained from patients undergoing bipolar hemiarthroplasty surgery for intra-capsular neck-of-femur fractures at the Mater Misericordiae University Hospital. Each femoral head was assessed intra-operatively to ensure it was non-arthritic.

The femoral head is the most proximal portion of the femur and is supported by the femoral neck. It forms approximately two-thirds of a sphere and articulates with the lunate surface of the acetabulum. It is covered with articular cartilage except over an ovoid depression, known as the *fovea capitis*, which serves as an attachment for the *ligamentum teres*.

2.2.2 Chemicals

All chemicals used in this research were of molecular biology, cell culture or analytical grade.

2.2.2.1 Iodine-based Contrast Agents

2.2.2.1.1 Iohexol

Iohexol (N, N´ - Bis (2, 3-dihydroxypropyl)-5-[N-(2, 3-dihydroxypropyl)-acetamido]-2, 4, 6-triiodoisophthalamide) is a nonionic, water-soluble, iodinated contrast agent with a molecular weight of 821.14 (iodine content 46.36%). In aqueous solution each tri-iodinated molecule remains undissociated. The chemical structure is shown in **Figure 2.1**. Iohexol (Omnipaque^{TM}, GE Healthcare, UK) was obtained from the Pharmacy Department of the Mater Misericordiae University Hospital at a concentration of 300 mg of organic iodine per ml (equivalent to 647 mg Iohexol per ml). This 300 mg/ml stock was used to make the relevant concentrations of Iohexol for each experiment.

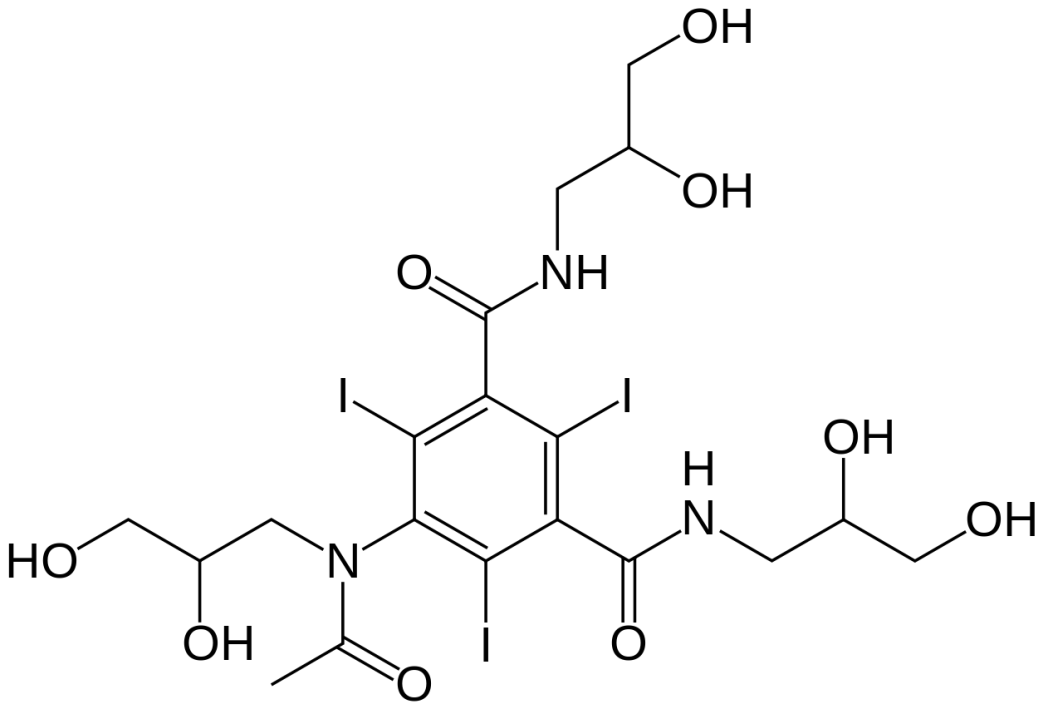

Figure 2.1. The molecular structure of Iohexol, $C_{19}H_{26}I_3N_3O_9$

2.2.2.1.2 Iopromide

Iopromide (1,3- Benzenedicarboxamide, N,N'-bis(2,3-dihydroxypropyl)-2,4,6-triiodo-5-[(methoxyacetyl)amino]-N-methyl) is a nonionic, water soluble, iodinated contrast agent with a molecular weight of 791.12 (iodine content 48.12%). The chemical structure is shown in **Figure 2.2**. Iopromide (Ultravist^{TM}, Bayer Limited, Ireland) was obtained from the Pharmacy Department of the Mater Misericordiae University Hospital at a concentration of 300 mg of organic iodine per ml (equivalent to 623.4 mg Iopromide per ml). This 300 mg/ml stock was used to make the relevant concentrations of Iopromide for each experiment.

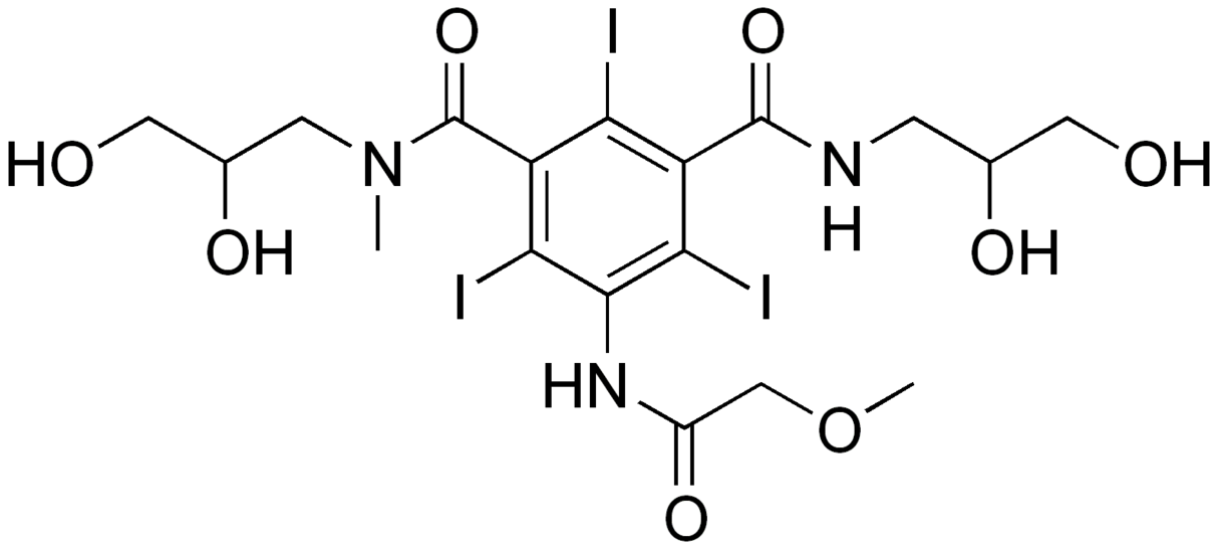

Figure 2.2. The molecular structure of Iopromide, $C_{18}H_{24}I_3N_3O_8$

2.2.2.2 Gadolinium-based Contrast Agents

2.2.2.2.1 Gadopentetate Dimeglumine

Gadopentetate dimeglumine (1-deoxy-1-(methylamino)-D-glucitol dihydrogen [N, N-bis [2-[bis (carboxymethyl) amino] ethyl] glycinato (5-)] gadolinate (2-) (2:1)) is the N-methylglucamine salt of the gadolinium complex of diethylenetriamine pentaacetic acid. It is an injectable contrast agent for MRI with a molecular weight of 938. Gadopentetate dimeglumine (Magnevist^{TM}, Bayer Limited, Ireland) was obtained from the Pharmacy Department of the Mater Misericordiae University Hospital at a concentration of 0.5 mmol/ml (equivalent to 469.01 mg/mL of gadopentetate dimeglumine). The chemical structure is shown in **Figure 2.3**. This 0.5 mmol/ml stock was used to make the relevant concentrations of Gadopentetate dimeglumine for each experiment.

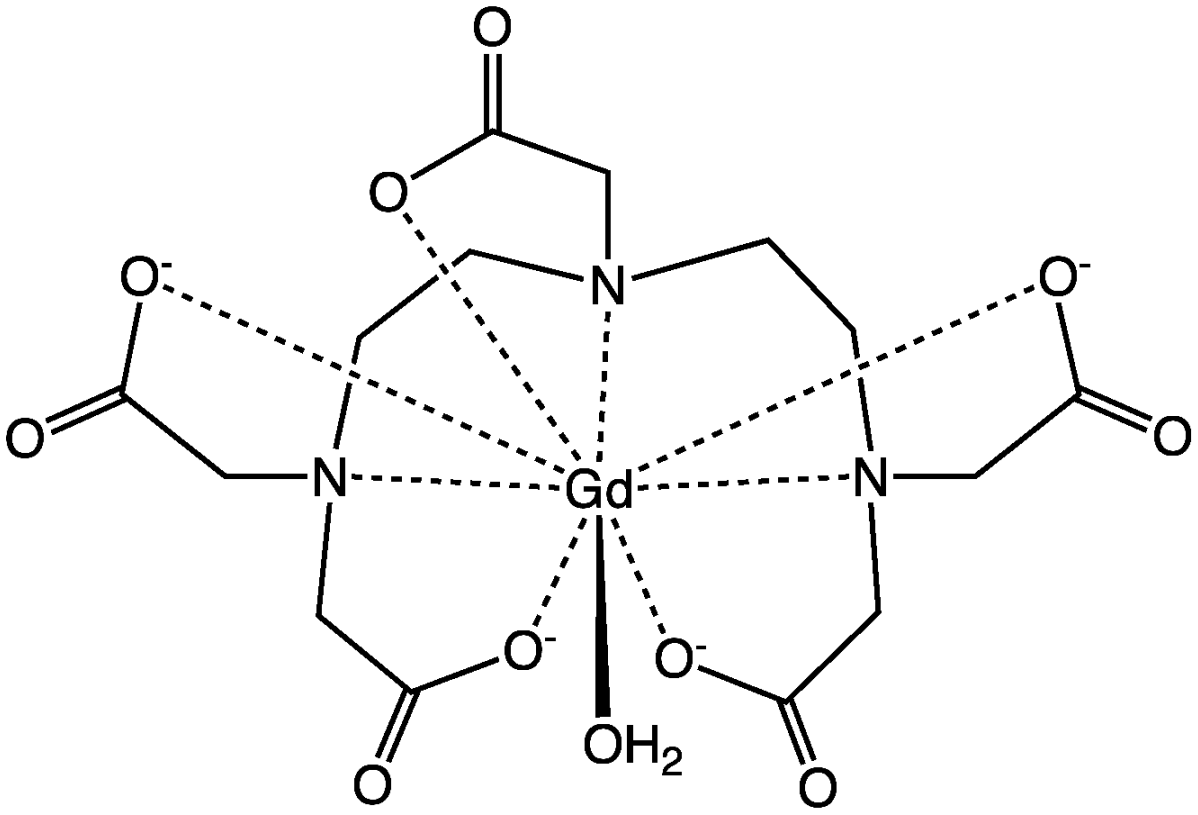

Figure 2.3. The molecular structure of Gadopentetate Dimeglumine, $C_{28}H_{54}GdN_5O_{20}$

2.2.2.2.2 Gadobenate Dimeglumine

Gadobenate dimeglumine (4RS-[4-carboxy-5, 8, 11-tris (carboxymethyl)-1phenyl-2-oxa-5, 8, 11-triazatridecan-13-oato (5-)] gadolinate (2-) dihydrogen compound with 1-deoxy-1(methylamino)-D-glucitol (1:2)) is an injectable contrast agent for MRI with a molecular weight of 1058.2. Gadobenate dimeglumine (Multihance^{TM}, Bayer Limited, Ireland) was obtained from the Pharmacy Department of the Mater Misericordiae University Hospital at a concentration of 0.5 mmol/ml (equivalent to 529 mg/mL of gadobenate dimeglumine). The chemical structure is shown in **Figure 2.4**. This 0.5 mmol/ml stock was used to make the relevant concentrations of Gadobenate dimeglumine for each experiment.

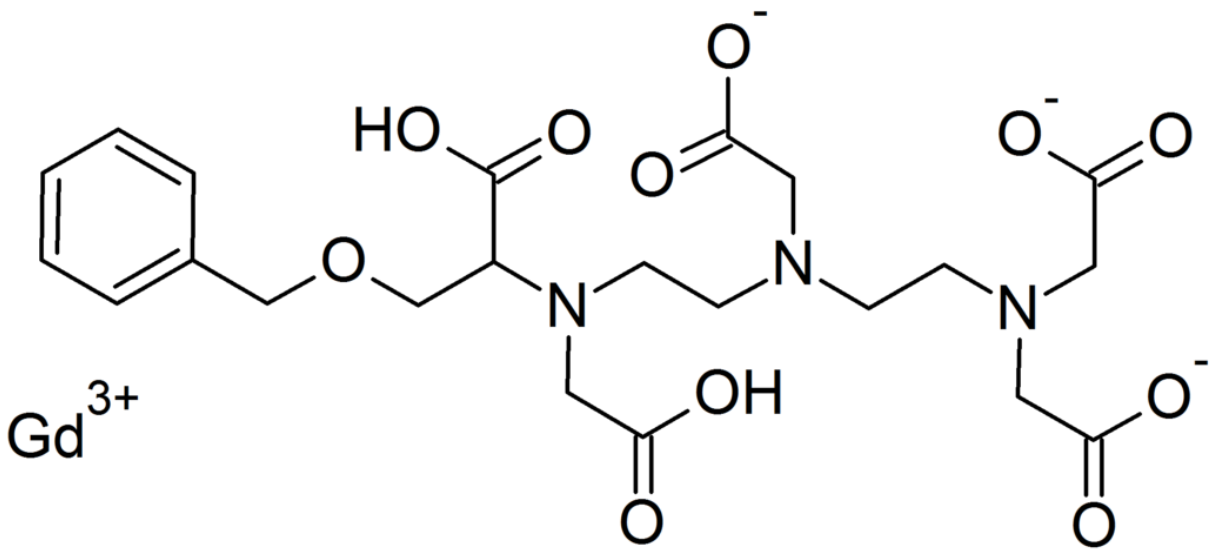

Figure 2.4. The molecular structure of Gadobenate Dimeglumine, $C_{22}H_{28}GdN_3O_{11}$

2.2.2.3 Local Anaesthetic

2.2.2.3.1 Lidocaine

Lidocaine (AstraZeneca, UK) is an amide local anaesthetic and was obtained from the Pharmacy Department of the Mater Misericordiae University Hospital in a soluble from at a concentration of 1%. This 1% stock was used to make the relevant concentrations of lidocaine for each experiment. The molecular structure of lidocaine is shown in **Figure 2.5**.

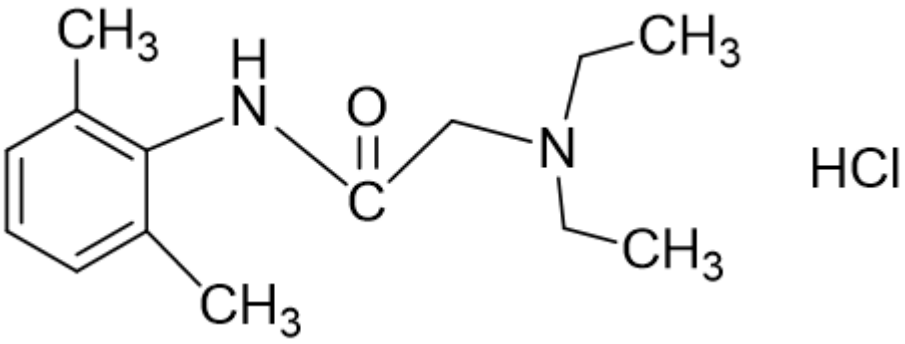

Figure 2.5. The molecular structure of Lidocaine, $C_{14}H_{22}N_2O$

2.2.3 Enzymes and Kits

Quantification of cellular viability was performed using (i) the Cell-Titer 96® AQueous One Solution Cell Proliferation Assay, also known as the MTS assay (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, PROMEGA, UK) and (ii) crystal violet staining (PROMEGA, UK).

The generation of cDNA from RNA was performed using a reverse transcription kit purchased from Sigma, UK. This included dNTP (D7295), oligo dT (O4387), 10X buffer (B1175) and the enzyme Avian reverse transcriptase (A4464).

Real-Time Polymerase Chain Reactions were performed with gene-specific primer sets and the Quantitect SYBR Green PCR Master Mix (Qiagen, UK).

2.3 Methods

All buffers and solutions for molecular biology experiments were prepared as outlined by Sambrook *et al* (1). Standard procedures for nucleic acid gel electrophoresis, ethidium bromide staining of electrophoresis gels and preparation of stock solutions were performed as described by Sambrook *et al* (1). Manufacturer's instructions were followed in all other protocols, unless otherwise stated.

2.3.1 Isolation of Primary Human Chondrocytes

Each femoral head was placed into a labelled sterile pot at the time of surgery by the operating surgeon or scrub nurse. The femoral head was then transferred to the laboratory in Dulbecco's Modified Eagle's Medium (DMEM; Sigma-Aldrich, UK) where it was used for the isolation of primary human chondrocytes.

In a fume hood, the femoral head was placed onto a sterile Petri dish and the articular cartilage was carefully screened for any gross evidence of pre-existing damage or degeneration such as softening, fibrillation or fissuring. Femoral heads were discarded if there was any anomaly.

The articular cartilage was sliced off and diced into 1-3 mm^3 pieces using two sterile scalpels. The diced cartilage was washed twice using DMEM to reduce the risk of microbial/fungal contamination. The diced cartilage was subsequently weighed in order to determine the wet weight and transferred to a 250 ml conical flask.

The cartilage was then subjected to sequential digestion with Pronase E (0.25%) for 1 hour, followed by Collagenase (0.025%) for 18 hours. Both digests were performed at 37^{o}C in a shaking water bath at 70 rpm and 60 rpm, respectively.

The following day, the digest was filtered through a 70 μm cell strainer and the resulting cell suspension was centrifuged at 220 G for 6 minutes.

The supernatant was discarded and the pellet re-suspended in 10 ml of DMEM and centrifuged at 220 G for 6 minutes. This step was performed twice.

After the second wash step, the pellet was re-suspended in 6 ml of DMEM and a cell count was performed (Section 2.3.4).

2.3.2 Cell Culture and Culture Conditions

Depending on cell number, the chondrocytes were plated into either a T25 or T75 flask. Cells were incubated at 37ºC in a humidified incubator containing 5% CO_2 for 7 days. During this time, the cell culture media (DMEM, 10% fetal bovine serum and 1% penicillin/streptomycin) was changed every 2 days (**Table 2.1**).

Once flasks reached 80% confluency, the cells were detached by trypsinisation, counted in a haemocytometer chamber, and seeded into 96-well plates (Section 2.3.3).

Confluent cultures were routinely checked for the presence of Collagen Type II to ensure that the chondrocytes studied had a normal phenotype (Section 2.3.7)

Table 2.1 Cell culture media volumes

	T25 Flask	**T75 Flask**
Day 1	7 ml	15 ml
Day 3	7 ml	15 ml
Day 5	9 ml	18 ml

2.3.3 Subculture of Cells

Once cells were microscopically judged to be of sufficient confluency (approximately 80% confluence), they were subcultured by trypsinisation.

From a 75 cm^2 tissue culture flask (SARSTEDT 83.181.002, vented cap), all culture media was discarded and cells were rinsed with 5 ml of sterile phosphate buffered solution (PBS) (P4417-100TAB, Sigma-Aldrich, UK) to remove any residual culture media. A volume of 5 ml of fresh trypsin-EDTA solution (1X) (T-3924, Sigma-Aldrich, UK) was added to the monolayer culture and the flask incubated at 37ºC for 5 minutes or until all the cells were seen to be detached from the flask surface by light microscopy. Next, 10 ml of growth culture medium was added to the cell suspension and the 15 ml mixture transferred to a sterile 15 ml universal container prior to being centrifuged at 1000 rpm for 5 minutes (Eppendorf

Centrifuge machine No: 5810). The trypsin-growth culture media was discarded and the resultant cell pellet was re-suspended in 4 ml of culture medium.

A 50 µL aliquot was set aside for cell counting.

Cells were seeded into a 96-well plates at a cell density of 1 x 10^4 cells per well (200 µL volume). The cells were allowed to grow for 48 hours before experimental treatment was undertaken.

All cell culture work was conducted in microbiological safety cabinets installed with high efficiency air filters (HEPA). All instruments were cleaned with 70% isopropanol and all sterile equipment used was previously steam autoclaved at 121ºC for 25 minutes. Sterile packed equipment were only opened within the safety cabinets after the packaging was previously cleaned using 70% isopropanol.

2.3.4 Cell Counting

Cell counting was performed using a Neubauerhaemocytometer slide. Trypan blue exclusion dye 0.4% (T-8154, Sigma-Aldrich, UK) was used to determine cell viability during counting. A 10 µL aliquot of trypan blue was added to a 50 µL cell suspension, mixed gently and incubated at room temperature for 2 minutes. A 10 µL sample from this mixture was added to the counting chamber of the haemocytometer and cells were visualized by light microscopy (NIKON Eclipse TS100). Viable cells remained clear while dead cells stained blue due to loss of membrane integrity. The number of cells in each of the four quadrants was obtained and the total number of cells was determined using the following formula:

$$(average\ number\ of\ viable\ cells) \times 1.2\ (dilution\ factor) \times 1 \times 10^4\ (area\ under\ cover\ slip) = number\ of\ viable\ cells/ml\ of\ suspension$$

2.3.5 Treatment

Culture media was aspirated and each well rinsed with 100 µL PBS.

Cells were then treated with 100 µL of varying concentrations of Iohexol, Iopromide, Gadopentetate Dimeglumine, Gadobenate Dimeglumine, Lidocaine or a combination thereof for 30 minutes, 45 minutes, 1 hour, 4 hours, 16 hours or 24 hours (as outlined in Sections 2.3.5.1, 2.3.5.2, 2.3.5.3 and 2.3.5.4). Each agent was tested both in isolation and as part of the clinical solution that is injected during joint arthrography (Section 1.2.2). Cells exposed to cell culture media or saline served as controls.

Each treatment was performed 5 times per 96-well plate and on primary human chondrocyte cultures from 6 separate femoral heads. Each treatment was therefore repeated 30 times in total (30 wells).

After the specified time period had elapsed, the treatment solution was removed, the wells rinsed with 100 µL PBS, and the cells were incubated again in 100 µL cell culture media for 24 hours.

Twenty-four hours after exposure, cell viability was assessed (Section 2.3.6).

2.3.5.1 Iodine Contrast Agents

2.3.5.1.1 Iohexol

Table 2.2 Iohexol treatment protocol

Iohexol

[*]Concentration *(mg/ml)*	**Time** *(hours)*			
	1	4	16	24
50	(5 Tests) x 6 PHC Cell Cultures	(5 Tests) x 6 PHC Cell Cultures	(5 Tests) x 6 PHC Cell Cultures	(5 Tests) x 6 PHC Cell Cultures
100	(5 Tests) x 6 PHC Cell Cultures	(5 Tests) x 6 PHC Cell Cultures	(5 Tests) x 6 PHC Cell Cultures	(5 Tests) x 6 PHC Cell Cultures
200	(5 Tests) x 6 PHC Cell Cultures	(5 Tests) x 6 PHC Cell Cultures	(5 Tests) x 6 PHC Cell Cultures	(5 Tests) x 6 PHC Cell Cultures

[*]Iohexol (300 mg/ml) in solution with cell culture media; PHC, Primary Human Chondrocyte

2.3.5.1.2 Iopromide

Table 2.3 Iopromide treatment protocol

Iopromide

[*]**Concentration** *(mg/ml)*	**Time** *(hours)*			
	1	4	16	24
50	(5 Tests) x 6 PHC Cell Cultures	(5 Tests) x 6 PHC Cell Cultures	(5 Tests) x 6 PHC Cell Cultures	(5 Tests) x 6 PHC Cell Cultures
100	(5 Tests) x 6 PHC Cell Cultures	(5 Tests) x 6 PHC Cell Cultures	(5 Tests) x 6 PHC Cell Cultures	(5 Tests) x 6 PHC Cell Cultures
200	(5 Tests) x 6 PHC Cell Cultures	(5 Tests) x 6 PHC Cell Cultures	(5 Tests) x 6 PHC Cell Cultures	(5 Tests) x 6 PHC Cell Cultures

[*]Iopromide (300 mg/ml) in solution with cell culture media; PHC, Primary Human Chondrocyte

2.3.5.2 Gadolinium Contrast Agents

2.3.5.2.1 Gadopentetate Dimeglumine

Table 2.4 Gadopentetate Dimeglumine treatment protocol

Gadopentetate Dimeglumine

[†]**Dilution** *(v:v)*	**Concentration** (mmol/L)	**Time** *(hours)*			
		1	4	16	24
1:300	1.7	(5 Tests) x 6 PHC Cell Cultures	(5 Tests) x 6 PHC Cell Cultures	(5 Tests) x 6 PHC Cell Cultures	(5 Tests) x 6 PHC Cell Cultures
1:200	2.5	(5 Tests) x 6 PHC Cell Cultures	(5 Tests) x 6 PHC Cell Cultures	(5 Tests) x 6 PHC Cell Cultures	(5 Tests) x 6 PHC Cell Cultures
1:100	5	(5 Tests) x 6 PHC Cell Cultures	(5 Tests) x 6 PHC Cell Cultures	(5 Tests) x 6 PHC Cell Cultures	(5 Tests) x 6 PHC Cell Cultures
1:50	10	(5 Tests) x 6 PHC Cell Cultures	(5 Tests) x 6 PHC Cell Cultures	(5 Tests) x 6 PHC Cell Cultures	(5 Tests) x 6 PHC Cell Cultures

[†]Gadopentetate Dimeglumine (0.5 mmol/ml) in solution with cell culture media; v, volume; PHC, Primary Human Chondrocyte

2.3.5.2.2 Gadobenate Dimeglumine

Table 2.5 Gadobenate Dimeglumine treatment protocol

Gadobenate Dimeglumine

†**Dilution** *(v:v)*	**Concentration** (mmol/L)	**Time** *(hours)*			
		1	4	16	24
1:300	1.7	(5 Tests) x 6 PHC Cell Cultures	(5 Tests) x 6 PHC Cell Cultures	(5 Tests) x 6 PHC Cell Cultures	(5 Tests) x 6 PHC Cell Cultures
1:200	2.5	(5 Tests) x 6 PHC Cell Cultures	(5 Tests) x 6 PHC Cell Cultures	(5 Tests) x 6 PHC Cell Cultures	(5 Tests) x 6 PHC Cell Cultures
1:100	5	(5 Tests) x 6 PHC Cell Cultures	(5 Tests) x 6 PHC Cell Cultures	(5 Tests) x 6 PHC Cell Cultures	(5 Tests) x 6 PHC Cell Cultures
1:50	10	(5 Tests) x 6 PHC Cell Cultures	(5 Tests) x 6 PHC Cell Cultures	(5 Tests) x 6 PHC Cell Cultures	(5 Tests) x 6 PHC Cell Cultures

†Gadobenate Dimeglumine (0.5 mmol/ml) in solution with cell culture media; v, volume; PHC, Primary Human Chondrocyte

2.3.5.3 MR Arthrogram Clinical Solution

Gadopentetate Dimeglumine 0.5 mmol/ml in a 1:200 *v:v* dilution (2.5 mmol/L) was used for the MR Arthrogram Clinical Solutions.

Table 2.6 MR Arthrogram Clinical Solution treatment protocol

Solution *(20 ml)*	**Time** *(minutes)*		
	30	45	60
19.9 ml [*]Saline + 0.1 ml [†]Gadopentetate Dimeglumine	(5 Tests) x 6 PHC Cell Cultures	(5 Tests) x 6 PHC Cell Cultures	(5 Tests) x 6 PHC Cell Cultures
10 ml Saline + 10 ml [‡]Lidocaine	(5 Tests) x 6 PHC Cell Cultures	(5 Tests) x 6 PHC Cell Cultures	(5 Tests) x 6 PHC Cell Cultures
9.95 ml Saline + 9.95 ml Lidocaine + 0.1 ml Gadopentetate Dimeglumine	(5 Tests) x 6 PHC Cell Cultures	(5 Tests) x 6 PHC Cell Cultures	(5 Tests) x 6 PHC Cell Cultures
20 ml Lidocaine	(5 Tests) x 6 PHC Cell Cultures	(5 Tests) x 6 PHC Cell Cultures	(5 Tests) x 6 PHC Cell Cultures

[*]Saline, 0.9% NaCl; [†]Gadopentetate Dimeglumine, 0.5 mmol/ml; [‡]Lidocaine, 1% Lidocaine; PHC, Primary Human Chondrocyte

2.3.5.4 Fluoroscopy / X-Ray/ CT Arthrogram Clinical Solution

Table 2.7 Fluoroscopy / X-Ray/ CT Arthrogram Clinical Solution treatment protocol

Iohexol 150 mg/ml was used for the Fluoroscopy / X-Ray/ CT Arthrogram Clinical Solutions

Solution *(20 ml)*	**Time** *(minutes)*		
	30	45	60
10 ml [*]Saline + 10 ml [†]Iohexol	(5 Tests) x 6 PHC Cell Cultures	(5 Tests) x 6 PHC Cell Cultures	(5 Tests) x 6 PHC Cell Cultures
10 ml Saline + 10 ml [‡]Lidocaine	(5 Tests) x 6 PHC Cell Cultures	(5 Tests) x 6 PHC Cell Cultures	(5 Tests) x 6 PHC Cell Cultures
10 ml Iohexol + 10 ml Lidocaine	(5 Tests) x 6 PHC Cell Cultures	(5 Tests) x 6 PHC Cell Cultures	(5 Tests) x 6 PHC Cell Cultures
20 ml Lidocaine	(5 Tests) x 6 PHC Cell Cultures	(5 Tests) x 6 PHC Cell Cultures	(5 Tests) x 6 PHC Cell Cultures

[*]Saline, 0.9% NaCl; [†]Iohexol, 300 mg/ml; [‡]Lidocaine, 1% Lidocaine; PHC, Primary Human Chondrocyte

2.3.6 Assessment of cell viability

2.3.6.1 Quantification of cell viability using the MTS assay

Cell viability was determined using the CellTiter 96® AQueous One Solution Cell Proliferation Assay which is also known as the MTS assay (PROMEGA, UK). The MTS assay is a colorimetric method for determining the number of viable cells in a culture. The solution contains a tetrazolium compound that produces a coloured formazan product when bio-reduced by cells. This product is soluble in tissue culture medium and absorbs light maximally at 490 nm. The compound is reduced due to the mitochondrial activity of the cell (2) and the amount of formazan that is produced is directly proportional to the number of viable cells in a culture (**Figure 2.6**).

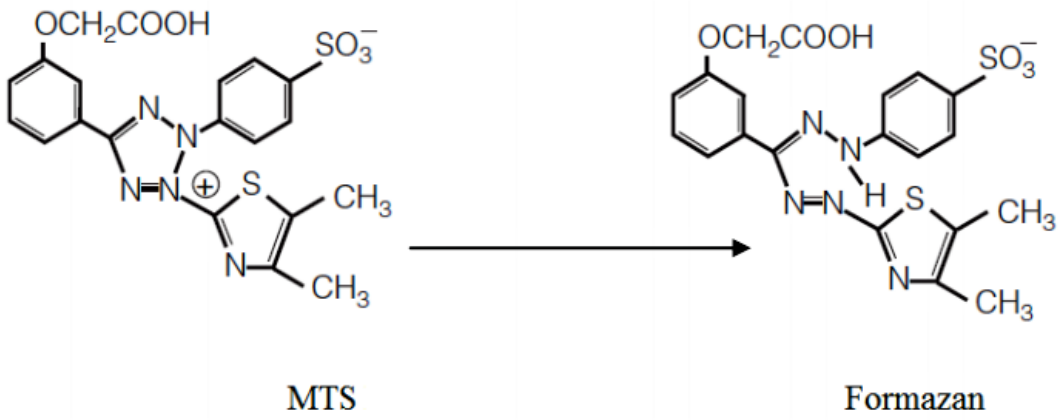

Figure 2.6. Structures of MTS tetrazolium and its formazan product

After the specified time period had elapsed, the treatment solution was removed, the wells rinsed with 100 µL PBS, and the cells were incubated again in 100 µL cell culture media for 24 hours. At this point, cell viability was assessed. A 20 µL aliquot of the MTS/PES (phenazine ethosulfate) solution was added to each well, and this was incubated for 2 hours at 37°C. The absorbance at 490 nm was recorded using a spectrophotometer (MOLECULAR DEVICES SPECTRAmax M2) and spectrometry data analysis software (MOLECULAR DEVICES – SoftMax Pro Software version 5.0.1).

2.3.6.2 Quantification of cell viability using crystal violet staining

Cell viability was also assessed by crystal violet staining. Crystal violet is a stain that binds to DNA and therefore can be used as a colorimetric method for determining cell density. The amount of dye taken up by the monolayer can be quantified in a spectrophotometer and is directly proportional to the number of viable cells.

Following treatment, the culture media was aspirated and each well was washed with 2 ml PBS. The cells were fixed with 96% ethanol solution and incubated for 10 minutes. The ethanol was then removed and the well allowed to dry for 1 to 2 minutes. Next, the well was stained with 0.05% crystal violet solution for 30 minutes. The stain was subsequently removed and rinsed three times with distilled H_2O to wash away excess dye. The cell-associated dye was then dissolved in 0.1% acetic acid/50% ethanol solution (2ml per well). Finally, 200 µl from each well was placed in a 96 well plate, placed into a spectrophotometer and the absorbance at 585 nm was recorded using a spectrometer (MOLECUALR DEVICES SPECTRAmax M2) and spectrometry data analysis software (MOLECULAR DEVICES – SoftMax Pro Software version 5.0.1).

2.3.7 RNA and DNA Preparation

To confirm chondral phenotype of the cells grown in culture, RNA samples were obtained from the cells and the level of chondrocyte-specific Collagen Type II was measured by reverse transcription-PCR.

2.3.7.1 Creation of a nuclease-free environment

As RNA is extremely susceptible to degradation by ribonucleases (RNases), it was essential to create a ribonuclease-free environment. Ribonucleases were eliminated from glassware, pipettes, tips and all solutions. Where possible, sterile disposable plastics were used instead of glassware. All glassware and other utensils were treated with 0.1% (v/v) diethyl pyrocarbonate (DEPC; Sigma-Aldrich, UK), a non-specific RNase inhibitor. Solutions were adjusted to 0.1% (v/v) DEPC, shaken thoroughly and left overnight in a fumehood; the following day the solutions were autoclaved to inactivate the DEPC. Sterile surgical gloves were worn throughout the entire procedure to minimize manual contamination.

2.3.7.2 RNA Isolation

Extraction of primary human chondrocyte RNA was performed using TRI (Total RNA Isolation) Reagent (T9424, Sigma-Aldrich, UK).

Initial cell culture was performed as outlined in Section 2.3.2. Once cells were microscopically judged to be of sufficient confluency (approximately 80% confluence), they were subcultured by trypsinisation (Section 2.3.3). Cells were seeded into 6-well plates at a cell density of 1 x 10^5 cells per well (2 mL volume). The cells were allowed to grow for 48 hours before phenotypic characterization was undertaken.

Supernatant was removed from the cells in cell culture and a 600 µL aliquot of ice-cold TRI Reagent added to each well. The plates were gently swirled to mix the reagent with the monolayer. The mixture was then pipetted gently several times to form a homogenous lysate. The resulting cell lysate from each pair of wells was combined and removed to a pre-chilled 1.5 ml Eppendorf™ conical tube and used for the isolation of RNA. This initial cell lysate was stored at -80°C until use (within one week).

2.3.7.2.1 Phase Preparation

After thawing, 0.24 ml of Chloroform was added to the lysate and the solution shaken vigorously for 15 seconds. The resulting mixture was subsequently stored at room temperature for 15 minutes and centrifuged at 12,000*g* for 15 minutes at 4°C. This resulted in a three-phase mixture: a red organic phase (containing protein), an interphase (containing DNA) and a colourless upper aqueous phase (containing RNA). The aqueous phase was pipetted into a new 1.5 ml Eppendorf™ conical tube

2.3.7.2.2 RNA Precipitation

A 150 µL aliquot of isopropanol, followed by a 2 µL aliquot of glycogen were added and the solution mixed gently. The glycogen acts as a carrier molecule and aids further precipitation, increasing the yield. This final solution was then stored overnight at -80°C.

The following day, the solution was allowed to thaw on ice and centrifuged at 12,000*g* for 10 minutes at 4°C. The supernatant was removed and the pellet (RNA-rich) was washed by vortexing with 1.4 ml of ice-cold 75% ethanol, followed by centrifugation at 12,000*g* for 10 minutes at 4°C. The supernatant was discarded and the resultant pellet was air-dried at room temperature for 10 minutes, ensuring that the pellet did not dry completely. The pellet was

then re-suspended in 20 µL of DEPC-treated H_2O. To facilitate re-suspension of the RNA pellets, samples were incubated at 55-60^oC for 10 minutes. The samples were then stored at -80^oC.

2.3.7.3 Gel Electrophoresis

Routine inspection of RNA quality involved gel electrophoresis. RNA integrity was determined using agarose gel electrophoresis to assess the integrity of the 28S, 18S and 5S ribosomal RNA, and using this as an indicator of total RNA integrity.

A 1% agarose gel was prepared by mixing 0.5 g of agarose with 50 ml of 1X TAE buffer (40 ml Tris-acetate, 10 ml EDTA). 25 µL of ethidium bromide was added to the gel mix and swirled several times. The resulting gel was poured into a cast and allowed to set for 20 minutes.

In a new 1.5 ml Eppendorf^{TM} conical tube, an aliquot of RNA (2 µL) was mixed with 1µL of RNA loading buffer (R4268, Sigma-Aldrich, UK) and 9 µL of DEPC-treated H_2O. This solution was heated to 65^oC for 10 minutes, chilled on ice for 1 minute and applied to the 1% agarose gel. Electrophoresis was performed at 150 V for 15 minutes in 1X TAE running buffer. To analyse the separated RNA, fluorescent RNA bands were captured under UV light in a Gel Reader (ThermoFisher Scientific, UK).

2.3.7.4 Reverse transcription of RNA – cDNA generation

Reverse transcriptions were carried out in 10 µL reaction mixtures using 2 µL of total RNA as the template. An aliquot containing 1 µL of dNTP mix (D7295, Sigma-Aldrich, UK) was missed with 1 µL oligo dT (O4387, Sigma-Aldrich, UK) and 6 µL of DEPC-treated H_2O. This solution was briefly mixed and centrifuged. It was then placed in the thermal cycler at 70^oC for 10 minutes to denature the RNA structure and hence allow for more efficient reverse transcription. The resulting solution was allowed to chill on ice for 1 minute.

To the cooled solution, 0.5 µL of RT enzyme (A4464, Sigma-Aldrich Enhanced Avian Reverse Transcriptase, UK), 2 µL of 10X buffer (B1175, Sigma-Aldrich, UK) and 7.5 µL of DEPC-treated H_2O was added. This was briefly centrifuged, then incubated at 45^0C for 90 minutes, followed by 95^oC for 2 minutes to ensure complete inactivation of the enzyme.

The sample was stored at 4^{o}C until further analysis was performed. An aliquot of this reaction was later used as a template to perform real-time polymerase chain reaction (RT – PCR) with gene-specific primers and Quantitect SYBER Green PCR Master Mix (Qiagen, UK).

2.3.7.5 Polymerase Chain Reaction

2.3.7.5.1 Primer Design

Oligonucleotides were designed based on the sequences obtained from the qPrimerDepot database, a primer database for quantitative real time PCR (3). This database provides primers for 99.6% of human RefSeq genes. Primers used as part of this research included:

i. Collagen Type I (Forward & Reverse)

ii. Collagen Type II (Forward & Reverse)

iii. GAPDH (Forward & Reverse) for 'housekeeping'

Primers were custom made by Sigma-Genosys, Ltd. Lyophilized primers were dissolved in sterile, double de-ionized H_2O (dd H_2O) to make a stock solution of 100 pmol/ µL. Aliquots (100 µL) of working solutions at a concentration of 10 pmol/ µL were made to avoid repeated thawing and freezing of stock solutions. All primer solutions were stored at -20^{o}C.

Table 2.8 Forward and reverse primers used in real-time PCR analysis

Gene	Forward Primer	Reverse Primer
Collagen Type I	5'-CGGCTCCTGCTCCTCTTAG-3'	5'-CACACGTCTCGGTCATGGTA-3'
Collagen Type II	5'-GTGTCAGGGCCAGGATGTC-3'	5'-GCAGAGGACAGTCCCAGTGT-3'
GAPDH	5'-GAGTCAACGGATTTGGTCGT-3'	5'-TTGATTTTGGAGGGATCTCG-3'

2.3.7.5.2 PCR Reaction Components

PCR reaction mixtures consisted of a 12.5 µL final volume cocktail containing 6.25 µL 1X Quantitect SYPER Green PCR Master Mix (Qiagen, UK), 0.3 µM sense primer and 0.3 µM anti-sense primer and 0.5 µl cDNA. The reaction volume was brought up to 12.5 µL with nuclease-free H_2O.

2.3.7.5.3 PCR Programme

PCR reactions were performed on a Rotor-Gene 3000^{TM} Thermocycler. A general PCR programme consisted of an initial DNA denaturation step of 15 minutes at 95ºC followed by 45 cycles of the following sequence incubations: 15s denaturation at 95ºC, 30s primer annealing time at a temperature calculated specifically for a given primer set and a 30s extension period per kb of product at 72ºC. At the end of the 45 cycles, melting curve analysis was performed to assess the purity of the PCR products.

2.4 Statistical Analysis

All data was compiled on an encrypted Excel database (Microsoft, Redmond, WA, USA).

Normality of distribution was assessed by examination of skewness and kurtosis z-values, Kolmogorov-Smirnov and Shapiro-Wilk tests and through visual inspection of histograms, box plots and normal Q-Q plots. In order to determine if cell viability was affected in either a time-dependent or dose-dependent fashion, a one-way analysis of variance (ANOVA) was used for parametric data, while a Kruskal-Wallis test was used for non-parametric data. Post-hoc analysis was performed in each case using a Bonferroni correction. A P value < 0.05 was considered statistically significant.

All statistical analysis was carried out in IBM SPSS Statistics for Windows, version 26 (IBM Corp., Armonk, N.Y., USA).

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3. Cui W, Taub DD, Gardner K. qPrimerDepot: a primer database for quantitative real time PCR. Nucleic Acids Res. 2007;35(Database issue):D805-9.

Chapter 3

Results

3.1 The isolation of primary human chondrocytes from patient femora

The isolation of primary human chondrocytes from the femoral heads of patients undergoing hip bipolar hemiarthroplasty is well-described (1-4), as is the use of such cells to investigate the effect of various therapies (5-8).

38 femoral heads from 38 patients (15 male and 23 female) were retrieved during the research period, with a median patient age of 74 years (range, 67 – 88 years). Chondrocyte cultures generated from 4 of the collected femoral heads did not grow well and were therefore discarded. No culture was affected by bacterial contamination. This lead to the cartilage from 3 femoral heads being used to optimise a protocol for the isolation of primary human chondrocytes, while final experiments were conducted using the cartilage of 31 femoral heads.

To optimise a protocol for the isolation of primary human chondrocytes, cartilage was removed from the femoral heads of three patients, diced into small chips and the wet weight obtained (Section 2.3.1). The cartilage chips were then divided into three equal portions and digested using one of the following three protocols.

Protocol 1 involved cartilage digestion in a 0.1% collagenase solution overnight in a shaking water bath at 37°C. While this protocol resulted in high cell yields, these cells did not subsequently lead to healthy chondrocyte cultures. Immediately post-digestion (Day 0), large amounts of debris were noted both in the cell cultures and surrounding some of the cells, indicating that the extracellular matrix had not been completely digested. Increasing the incubation time did not resolve this problem.

Protocol 2 involved the addition of 0.1% pronase to the digest for one hour followed by 24 hours digestion in the original 0.1% collagenase solution. Although the cell yields obtained during this method were less than in Protocol 1, the cells appeared healthier and adhered to the plastic culture flasks more readily.

For Protocol 3, the collagenase concentration was reduced to 0.025% and the pronase solution increased to 0.25% (9). This lead to the highest cell yield at Day 0 (**Table 3.1**), and microscopically the cells appeared healthier with complete digestion of the surrounding extracellular matrix (**Figure 3.1**).

After 7 days in culture (Section 2.3.2), the cells were imaged again to analyse differences in adherence and growth between the digestion protocols. The cells isolated using Protocol 1

continued to show unhealthy cultures and minimal growth. Although the cultures generated using Protocol 2 showed an increase in the number of cells, many of the cells looked irregular and unhealthy. In contrast, cultures generated using Protocol 3 contained healthy cells, approaching confluency (**Table 3.2** and **Figure 3.2**).

As Protocol 3 (0.25% pronase and 0.025% collagenase) resulted in the highest cell yields, with cultures that appeared the healthiest and representative of the chondrocyte phenotype, this isolation protocol was used in all subsequent sample processing.

Cell yields following sub-culturing of the cartilage (Section 2.3.3) used in the final experiments are given in **Table 3.3**.

Table 3.1 Cell yields (Day 0) using three different chondrocyte isolation protocols

	Protocol 1 (0.1% Collagenase)	**Protocol 2** (0.1% Pronase and 0.1% Collagenase)	**Protocol 3** (0.25% Pronase and 0.025% Collagenase)
Sample 1	5,500,000	3,200,00	7,000,000
Sample 2	2,400,000	2,150,000	3,108,000
Sample 3	1,400,000	995,000	2,000,500

Table 3.2 Comparison of chondrocyte cultures generated using three different chondrocyte isolation protocols

	Protocol 1 **(0.1% Collagenase)**	**Protocol 2** **(0.1% Pronase and 0.1% Collagenase)**	**Protocol 3** **(0.25% Pronase and 0.025% Collagenase)**
Day 0	Unhealthy cells Large amount of debris	Healthy cells Some debris	Healthy cells
Day 7	Unhealthy cells Minimal growth	Healthy cells mixed with abnormal cells	Healthy cells >80% confluency

Table 3.3 Cell yields following subculture of the cartilage used in the final experiments

Femoral Head	Cell Yield
1	1,587,000
2	1,362,000
3	698,666
4	875,600
5	1,548,000
6	1,297,200
7	768,000
8	1,554,666
9	850,000
10	438,000
11	2,116,000
12	736,000
13	1,115,200
14	5,056,000
15	464,000
16	1,788,000
17	914,666
18	1,402,666
19	416,000
20	1,552,000
21	648,000
22	1,072,000
23	2,295,000
24	1,320,000
25	945,000
26	1,512,000
27	3,507,000
28	2,400,000
29	1,000,200
30	980,000
31	2,630,000

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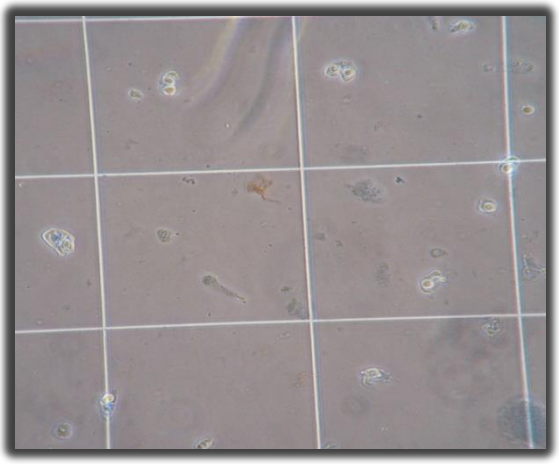

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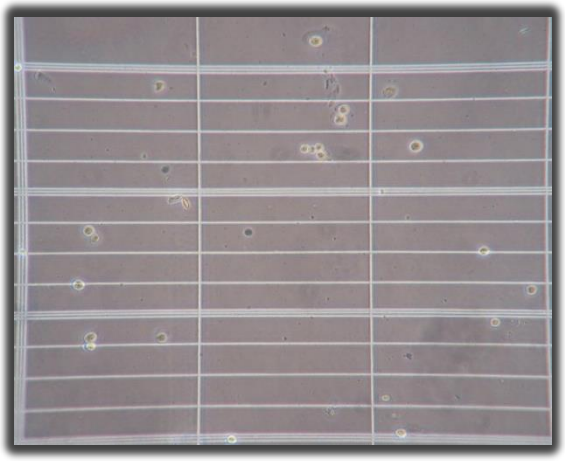

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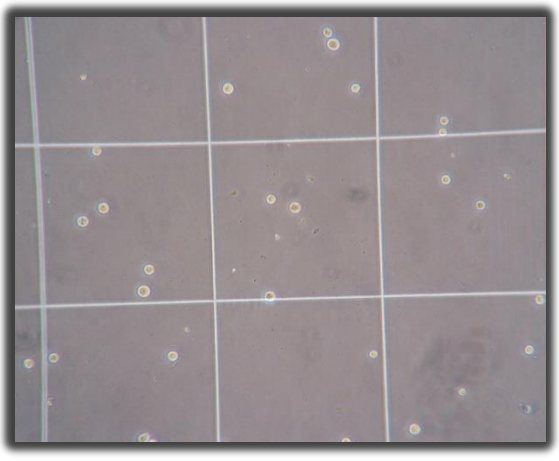
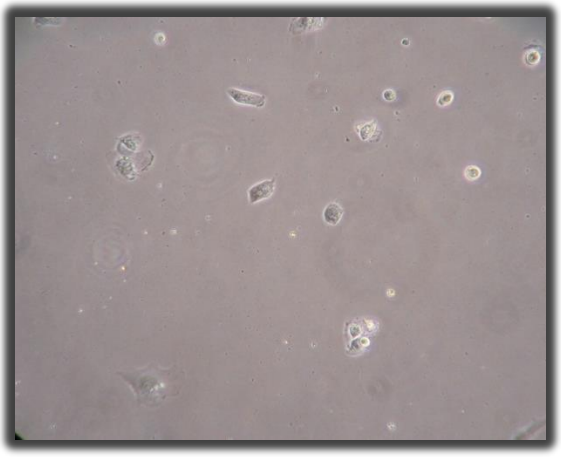


Figure 3.1 Phase contrast microscopy of primary human chondrocytes at Day 0 following isolation from cartilage using one of the three different isolation protocols: (A) Protocol 1 (0.1% collagenase); (B) Protocol 2 (0.1% pronase and 0.1% collagenase); (C) Protocol 3 (0.25% pronase and 0.025% collagenase). Images are shown at 20x magnification.



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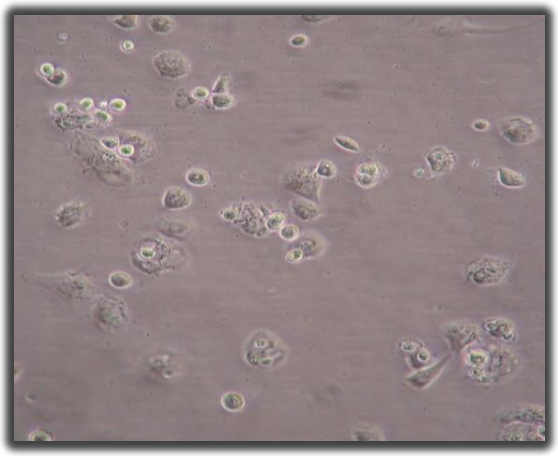

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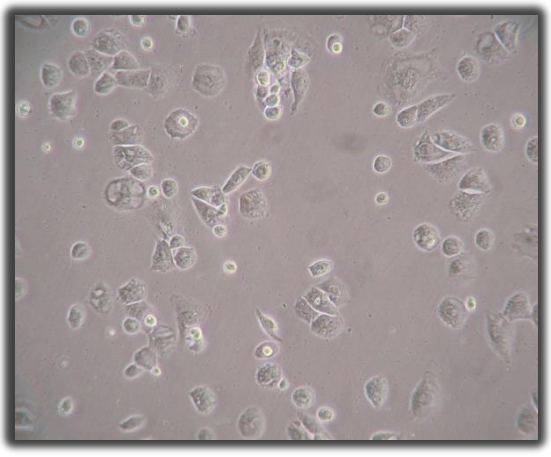

C

Figure 3.2 Phase contrast microscopy of primary human chondrocytes at Day 7 following isolation from cartilage using one of the three different isolation protocols: (A) Protocol 1 (0.1% collagenase); (B) Protocol 2 (0.1% pronase and 0.1% collagenase); (C) Protocol 3 (0.25% pronase and 0.025% collagenase). Images are shown at 20x magnification.

3.2 Phenotypic characterization of primary human chondrocytes

Before experiments were conducted with cells that were isolated using Protocol 3 (Section 3.1), verification of the chondrocyte phenotype was necessary. This was achieved using a combination of phase-contrast microscopy and real-time PCR.

Firstly, the morphology of freshly isolated chondrocyte cultures was examined using phase-contrast microscopy. Cell cultures were found to exhibit typical chondrocyte morphology, with a rounded or polygonal shape and a granular cytoplasm,

Secondly, the expression of Collagen Type II, a maker of differentiated chondrocytes was measured using real-time PCR. RNA was extracted from chondrocytes cultured to Day 7. This RNA was then converted to cDNA and used as a template in real-time PCR (Section 2.3.7). The results of real-time PCR analysis were expressed in terms of the cycle threshold (Ct) value, with the Ct value being inversely proportion to the expression of the gene. Therefore, the lower the Ct value, the higher the gene expression. At Day 7, Collagen Type II was highly expressed as compared to Collagen Type I (**Figure 3.3**). This finding indicated that the cells isolated from the femoral heads using Protocol 3 were chondrocytes, that the cells had not started to de-differentiate and that the cells were suitable for use in the next set of experiments.

Phenotypic characterization of primary human chondrocytes was routinely performed throughout the study.

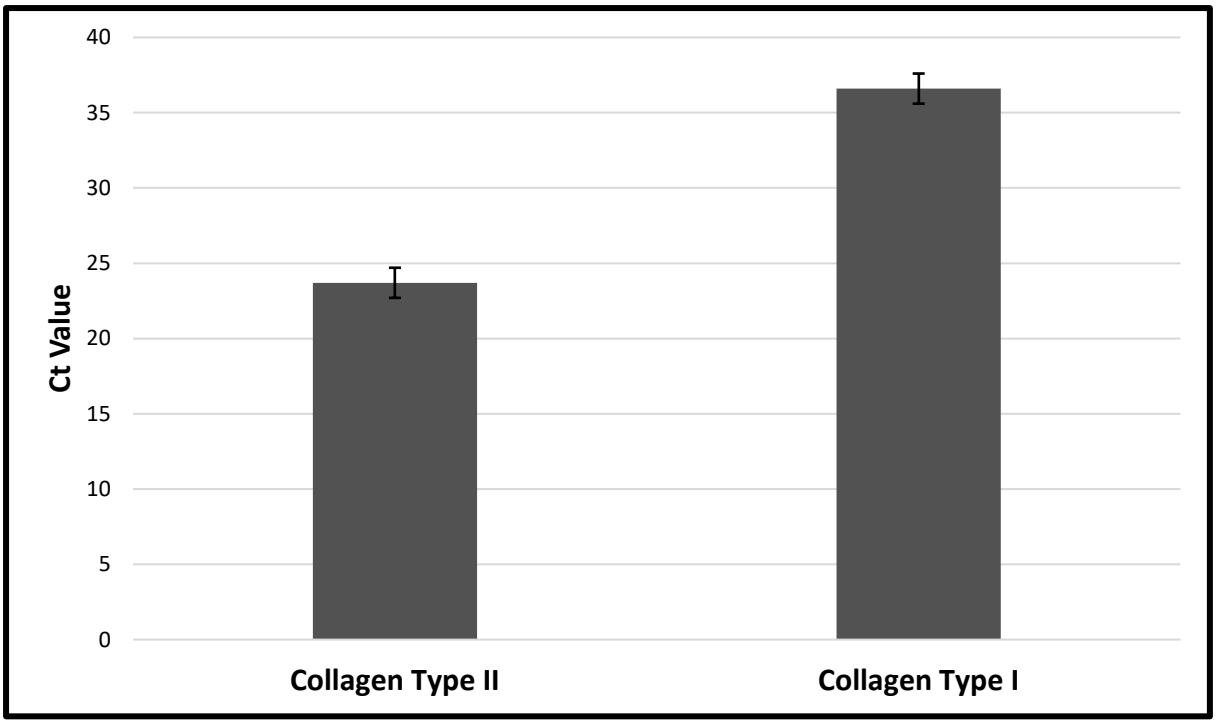

Figure 3.3 Bar graph depicting the expression of Collage Type II and Collagen Type I in primary human chondrocytes at Day 7. The cycle threshold (Ct) value is inversely proportional to gene expression.

3.3 The effect of Iohexol on primary human chondrocytes

To investigate the concentration-dependent and time-dependent effect of Iohexol on primary human chondrocyte cell viability, cells were treated with varying concentrations of Iohexol (either 50 mg/ml, 100 mg/ml or 200 mg/ml) for 1 hour, 4 hours, 16 hours and 24 hours (Section 2.3.5.1.1). Cells exposed to cell culture media served as the controls. Cell viability was then assessed using the MTS assay (Section 2.3.6.1). All data represents the results of six independent experiments.

3.3.1 Concentration-dependent effect of Iohexol on primary human chondrocytes

3.3.1.1 Iohexol: 1 Hour

A Shapiro-Wilks test and Kolmogorov-Smirnov test ($p > 0.05$) and a visual inspection of their histograms, normal Q-Q plots and box plots showed that cell viability scores were approximately normally distributed for each concentration, with a skewness of 0.97 (SE = 0.91) and a kurtosis of -0.28 (SE = 2.0) for 50 mg/ml, a skewness of -1.27 (SE = 0.85) and a kurtosis of 1.46 (SE = 1.74) for 100 mg/ml and a skewness of 0.78 (SE = 0.91) and a kurtosis of 1.35 (SE = 2.0) for 200 mg/ml.

At 1 hour there was no significant difference in cell viability between the media control and an Iohexol concentration of either 50 mg/ml (p = 1.0; 95% CI, -6.0 - 7.1), 100 mg/ml (p = 1.0; 95% CI, -5.9 – 6.5) or 200 mg/ml (p = 1.0; 95% CI, -3.5 – 9.5).

There was no significant concentration-dependent effect of Iohexol on cell viability ([50 mg/ml – 100 mg/ml, p = 1.0; 95% CI, -5.9 – 7.6], [50 mg/ml – 200 mg/ml, p = 0.92; 95% CI, -3.5 – 10.6], [100 mg/ml – 200 mg/ml, p = 1.0; 95% CI, -4.1 – 9.4]).

Table 3.4 Effect of treatment with varying concentrations of Iohexol for 1 hour on cell viability of primary human chondrocytes using the MTS assay. Data represents six independent experiments

	50 mg/ml	**100 mg/ml**	**200 mg/ml**	***p*-value**
Mean	100.6	99.7	97.0	
95% CI	96.8 – 104.4	97.1 – 102.4	88.1 – 105.9	
SD	3.1	2.5	7.1	
Range	97.8 – 105.2	95.2 – 102.0	88.6 – 107.9	
				p = 0.46

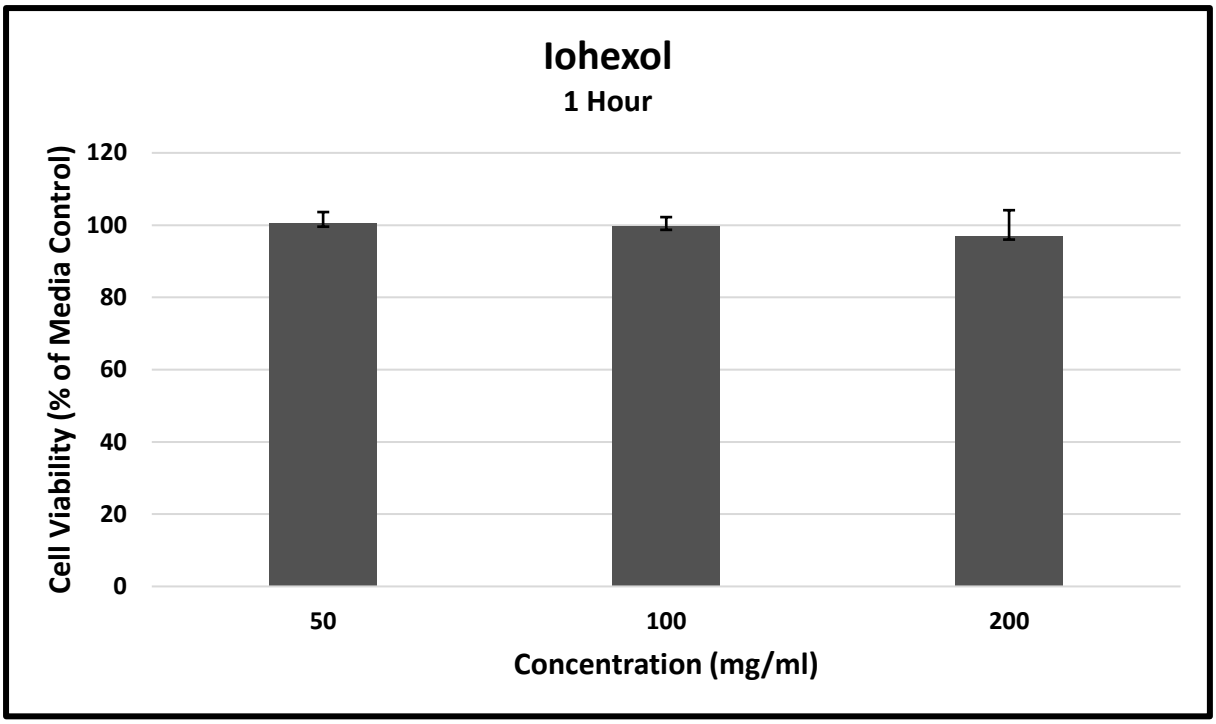

Figure 3.4 Bar graph illustrating the effect of treatment with varying concentrations of Iohexol for 1 hour on cell viability of primary human chondrocytes using the MTS assay. Data represents six independent experiments

3.3.1.2 Iohexol: 4 Hours

A Shapiro-Wilks test and Kolmogorov-Smirnov test ($p > 0.05$) and a visual inspection of their histograms, normal Q-Q plots and box plots showed that cell viability scores were approximately normally distributed for each concentration, with a skewness of 0.01 (SE = 0.85) and a kurtosis of -0.45 (SE = 1.74) for 50 mg/ml, a skewness of -0.62 (SE = 0.91) and a kurtosis of -0.08 (SE = 2.0) for 100 mg/ml and a skewness of 1.2 (SE = 1.0) and a kurtosis of 0.93 (SE = 2.6) for 200 mg/ml.

At 4 hours there was no significant difference in cell viability between the media control and an Iohexol concentration of either 50 mg/ml (p = 1.0; 95% CI, -3.5 – 7.5), 100 mg/ml (p = 0.45; 95% CI, -2.1 – 9.5) or 200 mg/ml (p = 0.11; 95% CI, -7.9 – 11.5).

There was no significant concentration-dependent effect of Iohexol on cell viability ([50 mg/ml – 100 mg/ml, p = 1.0; 95% CI, -4.1 – 7.5], [50 mg/ml – 200 mg/ml, p = 0.71; 95% CI, -2.8 – 9.6], [100 mg/ml – 200 mg/ml, p = 1.0; 95% CI, -4.7 – 8.1]).

Table 3.5 Effect of treatment with varying concentrations of Iohexol for 4 hours on cell viability of primary human chondrocytes using the MTS assay. Data represents six independent experiments

	50 mg/ml	**100 mg/ml**	**200 mg/ml**	***p*-value**
Mean	98.0	96.3	94.6	
95% CI	95.9 – 100.2	89.0 – 103.6	92.7 – 97.8	
SD	2.0	5.9	2.2	
Range	95.2 – 100.9	87.8 – 103.1	89.2 – 97.8	
				p = 0.09

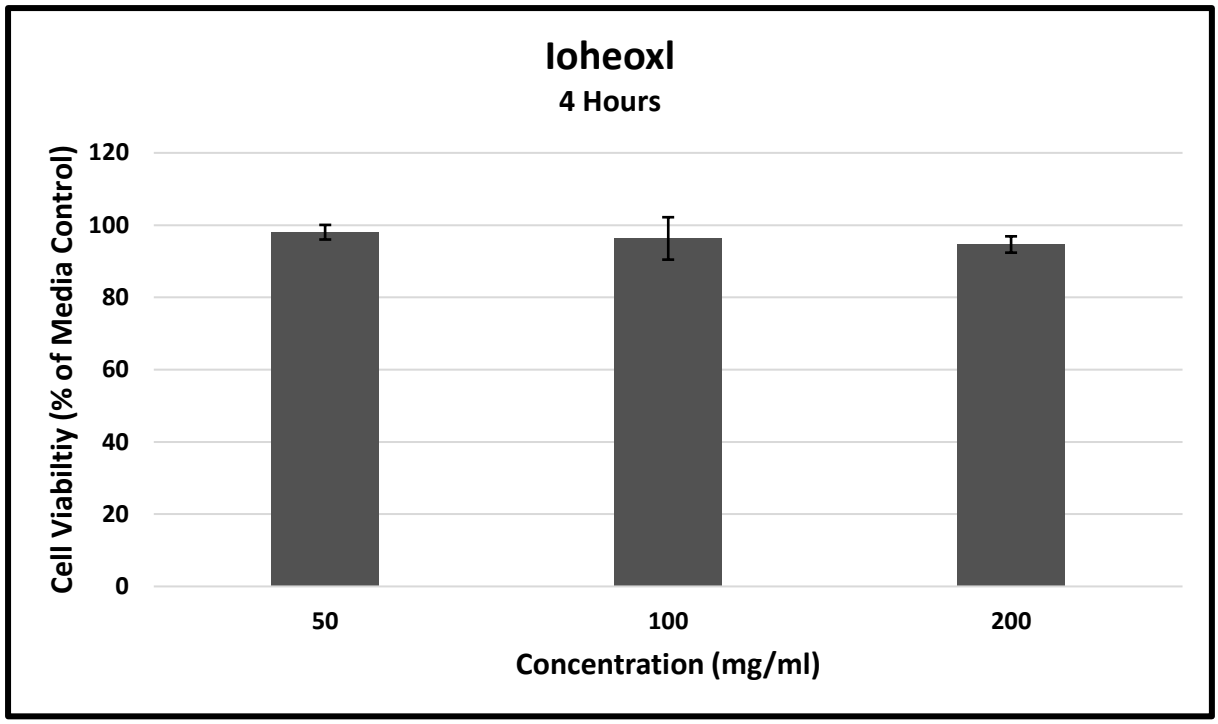

Figure 3.5 Bar graph illustrating the effect of treatment with varying concentrations of Iohexol for 4 hours on cell viability of primary human chondrocytes using the MTS assay. Data represents six independent experiments

3.3.1.3 Iohexol: 16 Hours

A Shapiro-Wilks test and Kolmogorov-Smirnov test ($p > 0.05$) and a visual inspection of their histograms, normal Q-Q plots and box plots showed that cell viability scores were approximately normally distributed for each concentration, with a skewness of 1.4 (SE = 0.91) and a kurtosis of 1.4 (SE = 2.0) for 50 mg/ml, a skewness of 1.1 (SE = 0.91) and a kurtosis of 2.1 (SE = 2.0) for 100 mg/ml and a skewness of -0.18 (SE = 0.91) and a kurtosis of -0.98 (SE = 2.0) for 200 mg/ml.

At 16 hours there was no significant difference in cell viability between the media control and an Iohexol concentration of 50 mg/ml ($p = 0.16$; 95% CI, -1.7 – 16.7). There was a significant difference in cell viability between the medial control and an Iohexol concentration of 100 mg/ml ($p = 0.003$; 95% CI, 4.0 – 22.4) and 200 mg/ml ($p = 0.02$; 95% CI, 1.4 – 19.8).

There was no significant concentration-dependent effect of Iohexol on cell viability ([50 mg/ml – 100 mg/ml, $p = 0.48$; 95% CI, -3.5 – 14.9], [50 mg/ml – 200 mg/ml, $p = 1.0$; 95% CI, -6.1 – 12.3], [100 mg/ml – 200 mg/ml, $p = 1.0$; 95% CI, -6.6 – 11.8]).

Table 3.6 Effect of treatment with varying concentrations of Iohexol for 16 hours on cell viability of primary human chondrocytes using the MTS assay. Data represents six independent experiments

	50 mg/ml	**100 mg/ml**	**200 mg/ml**	***p*-value**
Mean	92.5	86.8	89.4	
95% CI	81.8 – 103.2	83.6 – 90.0	85 – 93.8	
SD	8.6	2.6	3.6	
Range	85.4 – 106.5	84.0 – 91.0	84.7 – 93.9	
				$p = 0.003$

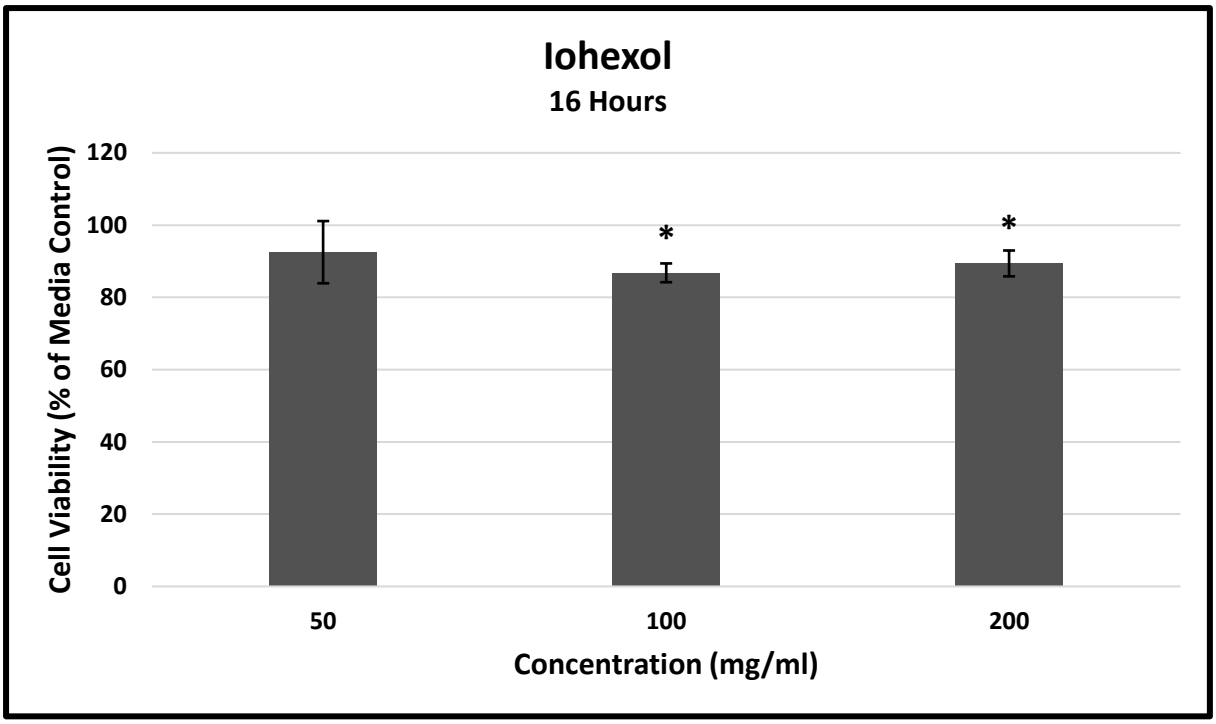

Figure 3.6 Bar graph illustrating the effect of treatment with varying concentrations of Iohexol for 16 hours on cell viability of primary human chondrocytes using the MTS assay. Data represents six independent experiments

*Statistically significant difference in cell viability from media control

3.3.1.4 Iohexol: 24 Hours

A Shapiro-Wilks test and Kolmogorov-Smirnov test ($p > 0.05$) and a visual inspection of their histograms, normal Q-Q plots and box plots showed that cell viability scores were approximately normally distributed for each concentration, with a skewness of 0.4 (SE = 0.8) and a kurtosis of 0.26 (SE = 1.59) for 50 mg/ml, a skewness of -1.7 (SE = 1.0) and a kurtosis of 3.2 (SE = 2.6) for 100 mg/ml and a skewness of -0.16 (SE = 0.91) and a kurtosis of -1.57 (SE = 2.0) for 200 mg/ml.

At 24 hours there was no significant difference in cell viability between the media control and an Iohexol concentration of either 50 mg/ml (p = 1.0; 95% CI, -7.1 – 15.8), 100 mg/ml (p = 0.97; 95% CI, -6.7 – 19.5) or 200 mg/ml (p = 0.55; 95% CI, -4.9 – 19.7).

There was no significant concentration-dependent effect of Iohexol on cell viability ([50 mg/ml – 100 mg/ml, p = 1.0; 95% CI, -10.2 – 14.3], [50 mg/ml – 200 mg/ml, p = 1.0; 95% CI, -8.4 – 14.5], [100 mg/ml – 200 mg/ml, p= 1.0; 95% CI, -12.1 – 14.1]).

Table 3.7 Effect of treatment with varying concentrations of Iohexol for 24 hours on cell viability of primary human chondrocytes using the MTS assay. Data represents six independent experiments

	50 mg/ml	**100 mg/ml**	**200 mg/ml**	***p*-value**
Mean	95.6	93.5	92.6	
95% CI	88.3 – 102.9	88.9 – 98.3	81.5 – 103.8	
SD	7.9	2.9	8.9	
Range	85.4 – 109.0	89.3 – 95.9	80.7 – 101.9	
				p = 0.33

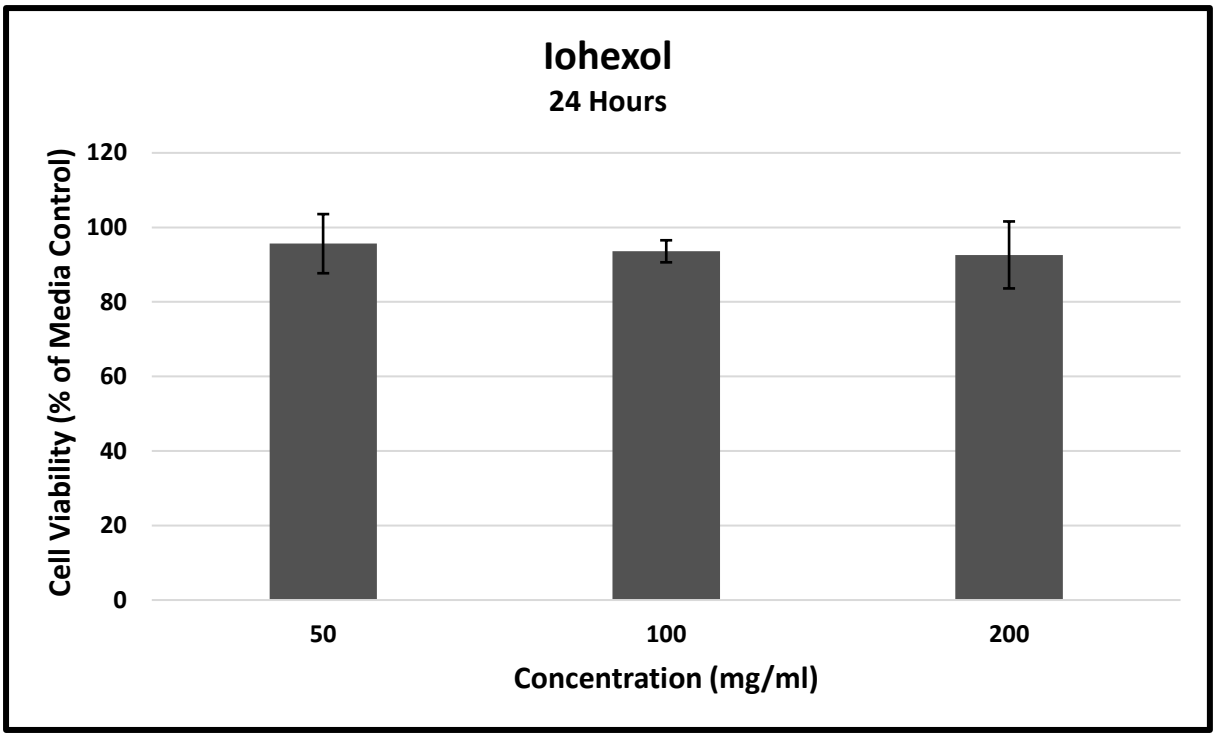

Figure 3.7 Bar graph illustrating the effect of treatment with varying concentrations of Iohexol for 24 hours on cell viability of primary human chondrocytes using the MTS assay. Data represents six independent experiments

3.3.2 Time-dependent effect of Iohexol on primary human chondrocytes

3.3.2.1 Iohexol: 50 mg/ml

A Shapiro-Wilks test and Kolmogorov-Smirnov test ($p > 0.05$) and a visual inspection of their histograms, normal Q-Q plots and box plots showed that cell viability scores were approximately normally distributed for each time point, with a skewness of 0.97 (SE = 0.91) and a kurtosis of -0.28 (SE = 2.0) for 1 hour, a skewness of 0.01 (SE = 0.85) and a kurtosis of -0.45 (SE = 1.74) for 4 hours, a skewness of 1.41 (SE = 0.91) and a kurtosis of 1.44 (SE = 2.0) for 16 hours and a skewness of 0.397 (SE = 0.79) and a kurtosis of 0.26 (SE = 1.59) for 24 hours.

There was no significant time-dependent effect of Iohexol 50 mg/ml on cell viability ([1 hour – 4 hours, $p = 1.0$; 95% CI, -8.5 – 13.6], [1 hour – 16 hours, $p = 0.33$; 95% CI, -3.5 – 19.6], [1 hour – 24 hours, $p = 1.0$; 95% CI, -5.8 – 15.6]), [4 hours – 16 hours, $p = 0.95$; 95% CI, -5.5 – 16.6]), [4 hours – 24 hours, $p = 1.0$; 95% CI, -7.8 – 12.6]) and [16 hours – 24 hours, $p = 1.0$; 95% CI, -7.6 – 13.8]).

Table 3.8 Effect of treatment with Iohexol 50 mg/ml for varying time periods on cell viability of primary human chondrocytes using the MTS assay. Data represents six independent experiments

	1 Hour	**4 Hours**	**16 Hours**	**24 Hours**	***p*-value**
Mean	100.6	98.0	92.5	95.6	
95% CI	96.8 – 104.4	95.9 – 100.2	81.8 – 103.2	88.3 – 102.9	
SD	3.1	2.0	8.6	7.9	
Range	97.8 – 105.2	95.3 – 100.9	85.4 – 106.5	85.4 – 109.0	
					$p = 0.231$

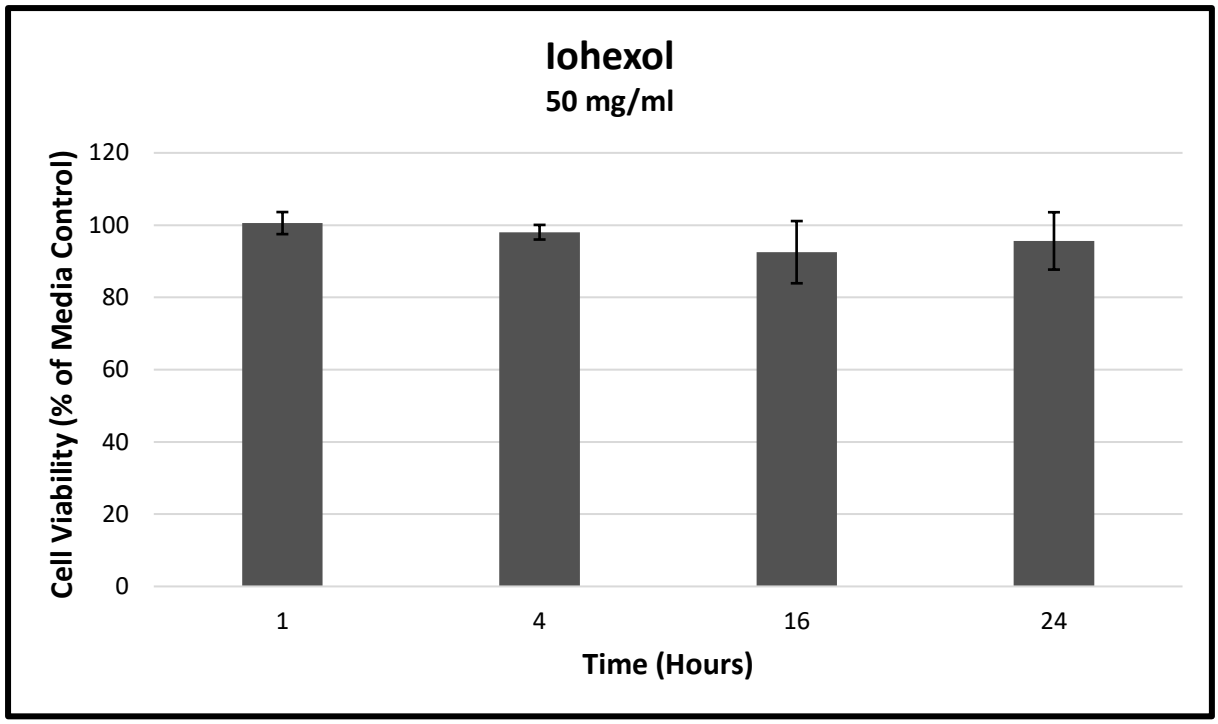

Figure 3.8 Bar graph illustrating the effect of treatment with Iohexol 50 mg/ml for varying time periods on cell viability of primary human chondrocytes using the MTS assay. Data represents six independent experiments

3.3.2.2 Iohexol: 100 mg/ml

A Shapiro-Wilks test and Kolmogorov-Smirnov test ($p > 0.05$) and a visual inspection of their histograms, normal Q-Q plots and box plots showed that cell viability scores were approximately normally distributed for each time point, with a skewness of -1.3 (SE = 0.85) and a kurtosis of 1.46 (SE = 1.74) for 1 hour, a skewness of -0.62 (SE = 0.91) and a kurtosis of -0.08 (SE = 2.0) for 4 hours, a skewness of 1.15 (SE = 0.91) and a kurtosis of 2.1 (SE = 2.0) for 16 hours and a skewness of -1.68 (SE = 1.0) and a kurtosis of 3.16 (SE = 2.62) for 24 hours.

There was a significant time-dependent effect of Iohexol 100 mg/ml on cell viability between 1 hour and 16 hours ($p < 0.001$; 95% CI, 6.1 – 19.7) and between 4 hours and 16 hours ($p = 0.006$; 95% CI, 2.4 – 16.6).

There was no significant difference between 1 hour and 4 hours ($p = 0.93$; 95% CI, -3.4 – 10.2), 1 hour and 24 hours ($p = 0.13$; 95% CI, -1.1 – 13.4), 4 hours and 24 hours ($p = 1.0$; 95% CI, -4.8 – 10.3) and 16 hours and 24 hours ($p = 0.09$; 95% CI, -0.75 – 14.3).

Table 3.9 Effect of treatment with Iohexol 100 mg/ml for varying time periods on cell viability of primary human chondrocytes using the MTS assay. Data represents six independent experiments

	1 Hour	**4 Hours**	**16 Hours**	**24 Hours**	***p*-value**
Mean	99.7	96.3	86.8	93.5	
95% CI	97.1 – 102.4	89.0 – 103.6	83.6 – 90.0	88.9 – 98.3	
SD	2.5	5.9	2.6	2.9	
Range	95.2 – 102.0	87.8 – 103.1	84.0 – 91.0	89.3 – 95.7	
					$p < 0.001$

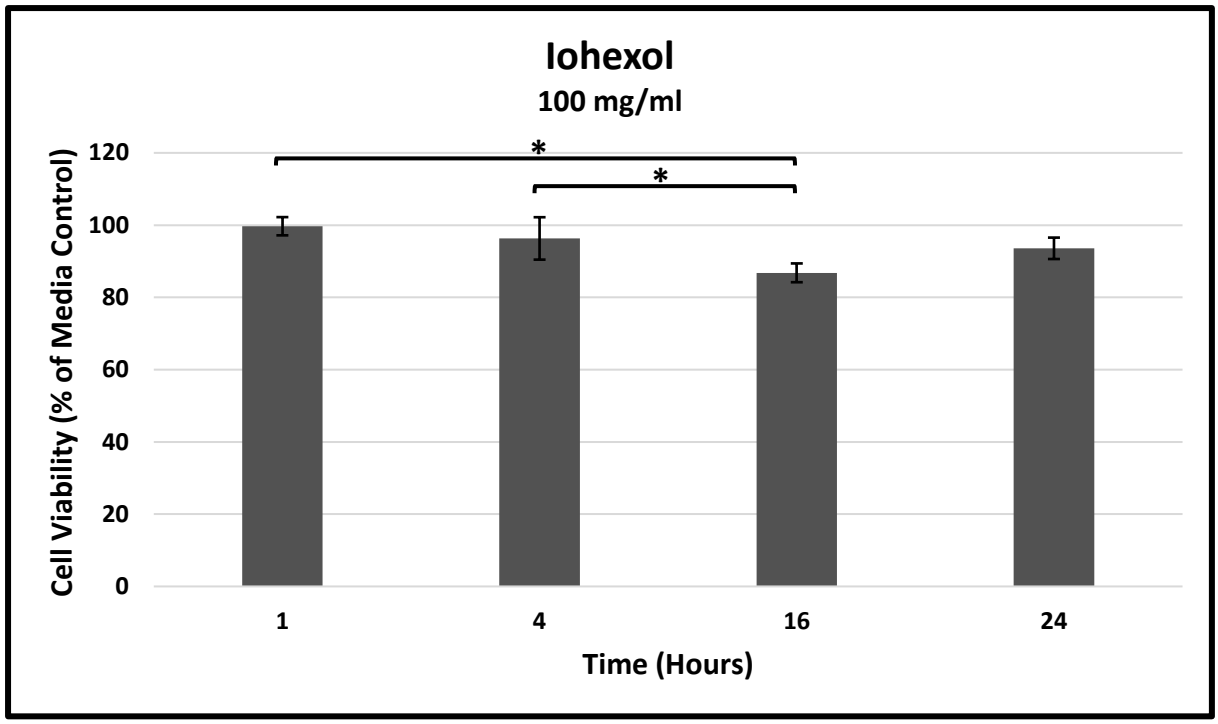

Figure 3.9 Bar graph illustrating the effect of treatment with Iohexol 100 mg/ml for varying time periods on cell viability of primary human chondrocytes using the MTS assay. Data represents six independent experiments

* Statistically significant difference in cell viability between time periods

3.3.2.3 Iohexol: 200 mg/ml

A Shapiro-Wilks test and Kolmogorov-Smirnov test ($p > 0.05$) and a visual inspection of their histograms, normal Q-Q plots and box plots showed that cell viability scores were approximately normally distributed for each time point, with a skewness of 0.78 (SE = 0.91) and a kurtosis of 1.35 (SE = 2.0) for 1 hour, a skewness of 1.2 (SE = 1.01) and a kurtosis of 0.93 (SE = 2.62) for 4 hours, a skewness of -0.18 (SE = 0.91) and a kurtosis of -0.96 (SE = 2.0) for 16 hours and a skewness of 0.16 (SE = 0.91) and a kurtosis of -1.57 (SE = 2.0) for 24 hours.

There was no significant time-dependent effect of Iohexol 200 mg/ml on cell viability ([1 hour – 4 hours, $p = 1.0$; 95% CI, -10.4 – 15.2], [1 hour – 16 hours, $p = 0.45$; 95% CI, -4.5 – 19.7], [1 hour – 24 hours, $p = 1.0$; 95% CI, -7.7 – 16.5]), [4 hours – 16 hours, $p = 1.0$; 95% CI, -7.6 – 18.0]), [4 hours – 24 hours, $p = 0.6$; 95% CI, -10.8 – 14.8]) and [16 hours – 24 hours, $p = 1.0$; 95% CI, -8.9 – 15.3]).

Table 3.10 Effect of treatment with Iohexol 200 mg/ml for varying time periods on cell viability of primary human chondrocytes using the MTS assay. Data represents six independent experiments

	1 Hour	**4 Hours**	**16 Hours**	**24 Hours**	***p*-value**
Mean	97.0	94.6	89.4	92.6	
95% CI	88.2 – 105.9	91.0 – 98.2	85.0 – 93.8	81.5 – 103.8	
SD	7.1	2.3	3.6	8.9	
Range	88.6 – 107.9	89.2 – 97.8	84.7 – 93.9	80.7 – 101.9	
					$p = 0.311$

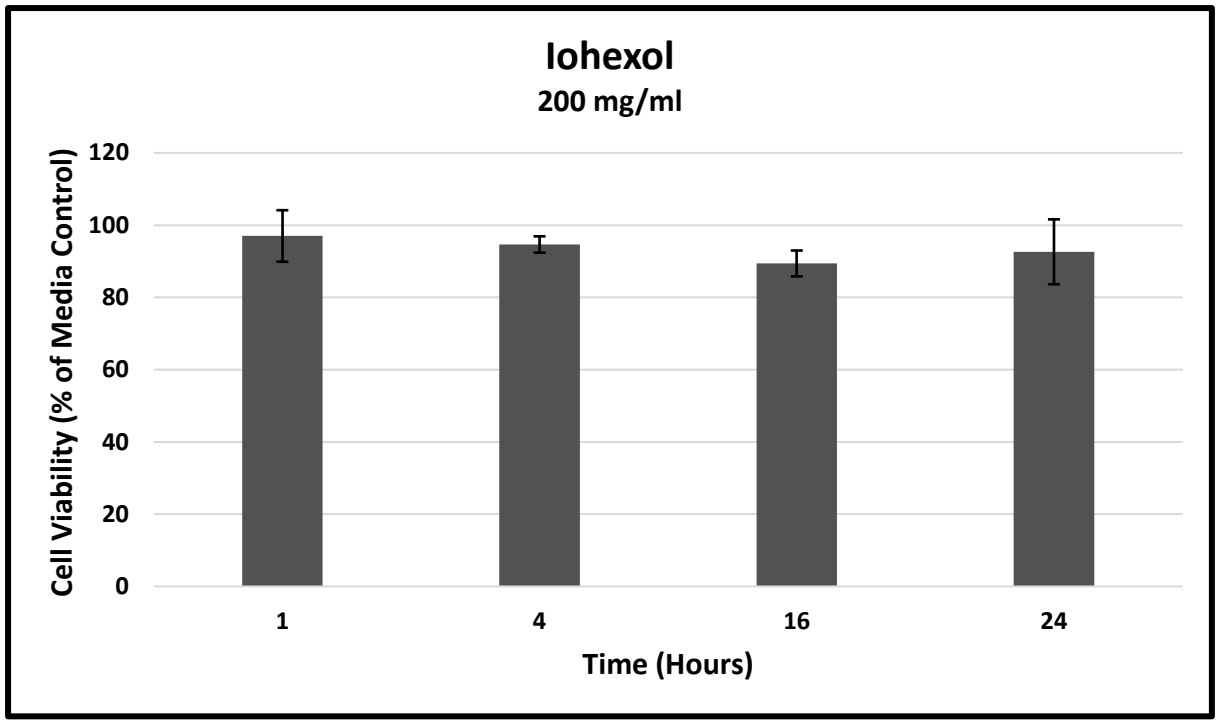

Figure 3.10 Bar graph illustrating the effect of treatment with Iohexol 200 mg/ml for varying time periods on cell viability of primary human chondrocytes using the MTS assay. Data represents six independent experiments

3.4. The effect of Iopromide on primary human chondrocytes

To investigate the concentration-dependent and time-dependent effect of Iopromide on primary human chondrocyte cell viability, cells were treated with varying concentrations of Iopromide (either 50 mg/ml, 100 mg/ml or 200 mg/ml) for 1 hour, 4 hours, 16 hours and 24 hours (Section 2.3.5.1.2). Cells exposed to cell culture media served as the controls. Cell viability was then assessed using the MTS assay (Section 2.3.6.1). All data represents the results of six independent experiments.

3.4.1 Concentration-dependent effect of Iopromide on primary human chondrocytes

3.4.1.1 Iopromide: 1 Hour

A Shapiro-Wilks test and Kolmogorov-Smirnov test ($p > 0.05$) and a visual inspection of their histograms, normal Q-Q plots and box plots showed that cell viability scores were approximately normally distributed for each concentration, with a skewness of -0.38 (SE = 0.91) and a kurtosis of -1.5 (SE = 2.0) for 50 mg/ml, a skewness of -0.5 (SE = 0.91) and a kurtosis of -1.1 (SE = 2.0) for 100 mg/ml and a skewness of 0.90 (SE = 0.91) and a kurtosis of 1.57 (SE = 2.0) for 200 mg/ml.

At 1 hour there was no significant difference in cell viability between the media control and an Iopromide concentration of either 50 mg/ml ($p = 1.0$; 95% CI, -7.4 – 17.9), 100 mg/ml ($p = 1.0$; 95% CI, -8.8 – 16.6) or 200 mg/ml ($p = 1.0$; 95% CI, -8.2 – 17.2).

There was no significant concentration-dependent effect of Iopromide on cell viability ([50 mg/ml – 100 mg/ml, $p = 1.0$; 95% CI, -11.3 – 14.1], [50 mg/ml – 200 mg/ml, $p = 1.0$; 95% CI, -11.9 – 13.5], [100 mg/ml – 200 mg/ml, $p = 1.0$; 95% CI, -12.1 – 13.3]).

Table 3.11 Effect of treatment with varying concentrations of Iopromide for 1 hour on cell viability of primary human chondrocytes using the MTS assay. Data represents six independent experiments

	50 mg/ml	**100 mg/ml**	**200 mg/ml**	***p*-value**
Mean	94.7	96.1	95.5	
95% CI	92.4 – 99.9	89.9 – 102.3	80.3 – 110.7	
SD	1.8	4.9	12.2	
Range	92.2 – 96.5	89.3 – 101.8	81.5 – 114.6	
				$p = 0.612$

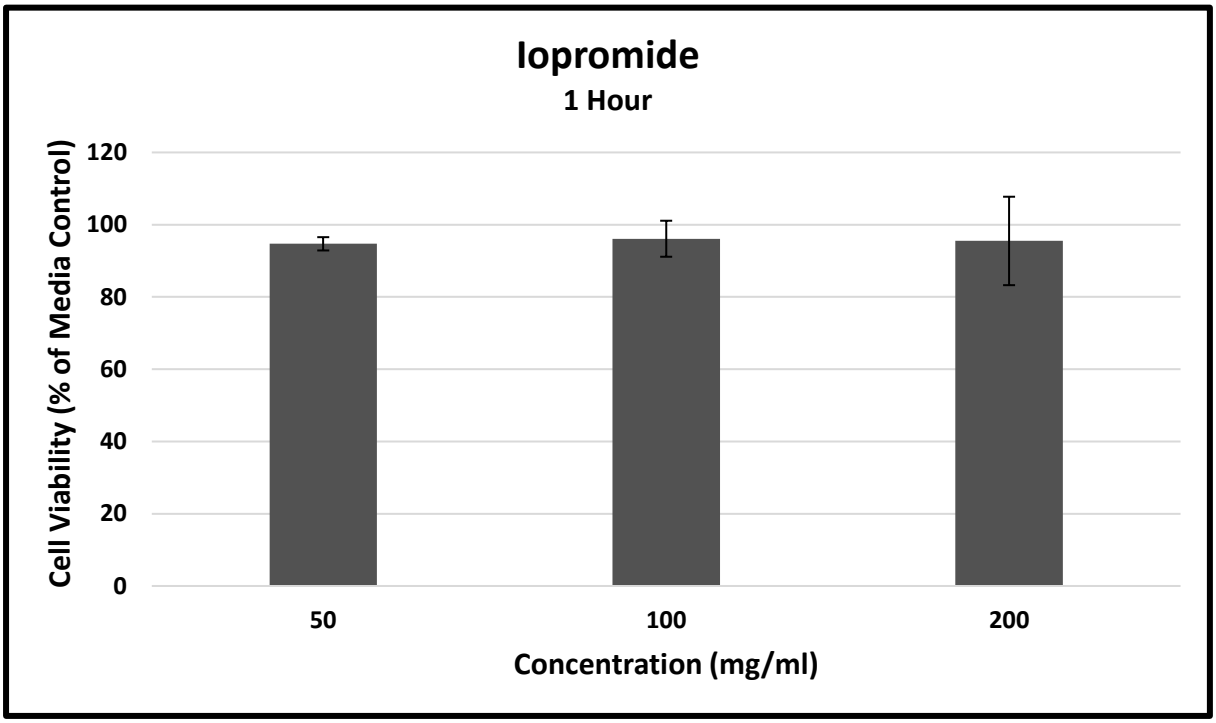

Figure 3.11 Bar graph illustrating the effect of treatment with varying concentrations of Iopromide for 1 hour on cell viability of primary human chondrocytes using the MTS assay. Data represents six independent experiments

3.4.1.2 Iopromide: 4 Hours

A Shapiro-Wilks test and Kolmogorov-Smirnov test ($p > 0.05$) and a visual inspection of their histograms, normal Q-Q plots and box plots showed that cell viability scores were approximately normally distributed for each concentration, with a skewness of 0.11 (SE = 0.85) and a kurtosis of -01.88 (SE = 1.74) for 50 mg/ml, a skewness of 0.48 (SE = 0.85) and a kurtosis of -1.88 (SE = 1.74) for 100 mg/ml and a skewness of -0.04 (SE = 0.69) and a kurtosis of -2.04 (SE = 1.33) for 200 mg/ml.

At 4 hours there was no significant difference in cell viability between the media control and an Iopromide concentration of either 50 mg/ml (p = 1.0; 95% CI, -5.9 – 6.9), 100 mg/ml (p = 0.17; 95% CI, -1.2 – 11.6) or 200 mg/ml (p = 0.06; 95% CI, 1.7 – 13.2).

There was no significant concentration-dependent effect of Iopromide on cell viability ([50 mg/ml – 100 mg/ml, p = 0.28; 95% CI, -1.7 – 11.1], [50 mg/ml – 200 mg/ml, p = 0.12; 95% CI, -1.2 – 12.7], [100 mg/ml – 200 mg/ml, p = 1.0; 95% CI, -3.5 – 7.9]).

Table 3.12 Effect of treatment with varying concentrations of Iopromide for 4 hours on cell viability of primary human chondrocytes using the MTS assay. Data represents six independent experiments

	50 mg/ml	**100 mg/ml**	**200 mg/ml**	***p*-value**
Mean	99.5	94.8	92.6	
95% CI	97.5 – 101.5	92.0 – 97.7	88.4 – 96.7	
SD	1.9	2.7	5.8	
Range	97.5 – 101.6	92.1 – 98.1	84.7 – 99.7	
				p = 0.2

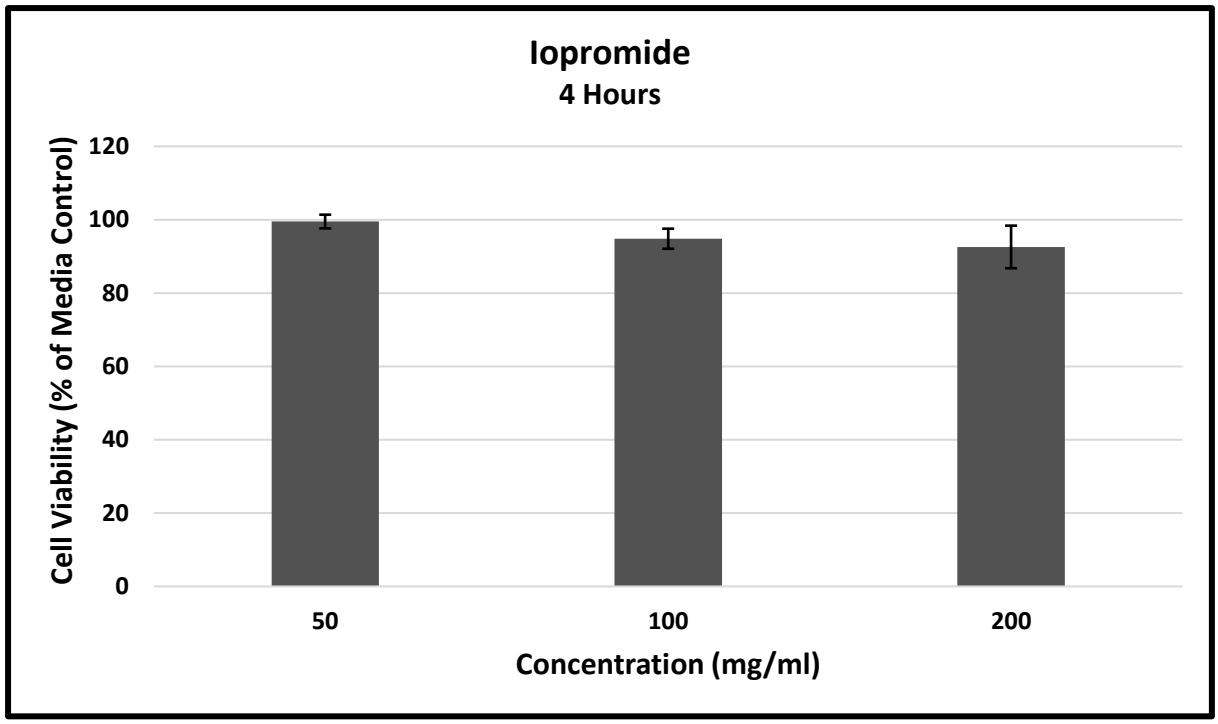

Figure 3.12 Bar graph illustrating the effect of treatment with varying concentrations of Iopromide for 4 hours on cell viability of primary human chondrocytes using the MTS assay. Data represents six independent experiments

3.4.1.3 Iopromide: 16 Hours

A Shapiro-Wilks test and Kolmogorov-Smirnov test ($p > 0.05$) and a visual inspection of their histograms, normal Q-Q plots and box plots showed that cell viability scores were approximately normally distributed for each concentration, with a skewness of 0.74 (SE = 0.91) and a kurtosis of 2.19 (SE = 2.0) for 50 mg/ml, a skewness of 0.54 (SE = 0.91) and a kurtosis of 0.66 (SE = 2.0) for 100 mg/ml and a skewness of 0.57 (SE = 0.91) and a kurtosis of -0.13 (SE = 2.0) for 200 mg/ml.

At 16 hours there was no significant difference in cell viability between the media control and an Iopromide concentration of 50 mg/ml ($p = 0.27$; 95% CI, -2.3 – 14.6) or 100 mg/ml ($p = 0.06$; 95% CI, -0.2 – 16.8). There was a significant difference in cell viability between the medial control and an Iopromide concentration of 200 mg/ml ($p = 0.03$; 95% CI, 6.3 – 10.6).

There was no significant concentration-dependent effect of Iopromide on cell viability ([50 mg/ml – 100 mg/ml, $p = 1.0$; 95% CI, -6.3 – 10.6], [50 mg/ml – 200 mg/ml, $p = 1.0$; 95% CI, -5.3 – 11.7], [100 mg/ml – 200 mg/ml, $p= 1.0$; 95% CI, -7.4 – 9.5]).

Table 3.13 Effect of treatment with varying concentrations of Iopromide for 16 hours on cell viability of primary human chondrocytes using the MTS assay. Data represents six independent experiments

	50 mg/ml	**100 mg/ml**	**200 mg/ml**	***p*-value**
Mean	93.9	91.7	90.7	
95% CI	86.3 – 101.4	84.5 – 98.9	87.0 – 94.3	
SD	6.1	5.8	2.9	
Range	86.3 – 103.3	84.7 – 100.2	87.4 – 94.9	
				$p = 0.02$

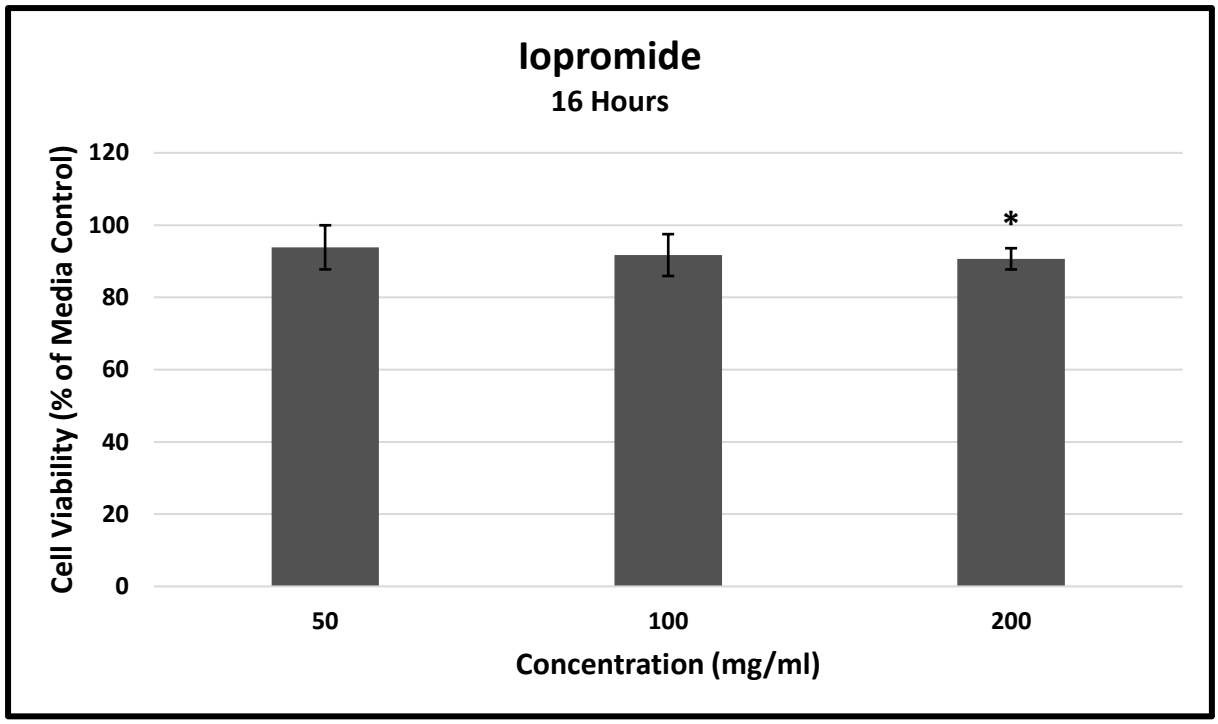

Figure 3.13 Bar graph illustrating the effect of treatment with varying concentrations of Iopromide for 16 hours on cell viability of primary human chondrocytes using the MTS assay. Data represents six independent experiments

*Statistically significant difference in cell viability from media control

3.4.1.4 Iopromide: 24 Hours

A Shapiro-Wilks test and Kolmogorov-Smirnov test ($p > 0.05$) and a visual inspection of their histograms, normal Q-Q plots and box plots showed that cell viability scores were approximately normally distributed for each concentration, with a skewness of 1.23 (SE = 1.01) and a kurtosis of 0.95 (SE = 2.61) for 50 mg/ml, a skewness of 1.7 (SE = 0.91) and a kurtosis of 3.08 (SE = 2.0) for 100 mg/ml and a skewness of -0.27 (SE = 0.91) and a kurtosis of -3.0 (SE = 2.0) for 200 mg/ml.

At 24 hours there was no significant difference in cell viability between the media control and an Iopromide concentration of 50 mg/ml ($p = 0.13$; 95% CI, -1.2 – 14.9). There was a significant difference between the media control and an Iopromide concentration of 100 mg/ml ($p = 0.001$; 95% CI, 5.3 – 20.65) and 200 mg/ml ($p = 0.002$; 95% CI, 4.3 – 19.6).

There was no significant concentration-dependent effect of Iopromide on cell viability ([50 mg/ml – 100 mg/ml, $p = 0.223$; 95% CI, 2.0 – 14.2], [50 mg/ml – 200 mg/ml, $p = 0.45$; 95% CI, 3.0 – 13.2], [100 mg/ml – 200 mg/ml, $p= 1.0$; 95% CI, 6.6 – 8.6]).

Table 3.14 Effect of treatment with varying concentrations of Iopromide for 24 hours on cell viability of primary human chondrocytes using the MTS assay. Data represents six independent experiments

	50 mg/ml	**100 mg/ml**	**200 mg/ml**	***p*-value**
Mean	93.1	87.0	88.0	
95% CI	86.3 – 90.7	83.4 – 90.7	80.5 – 95.6	
SD	4.3	2.9	6.1	
Range	89.5 – 99.1	84.8 – 92.0	81.6 – 94.2	
				$p < 0.001$

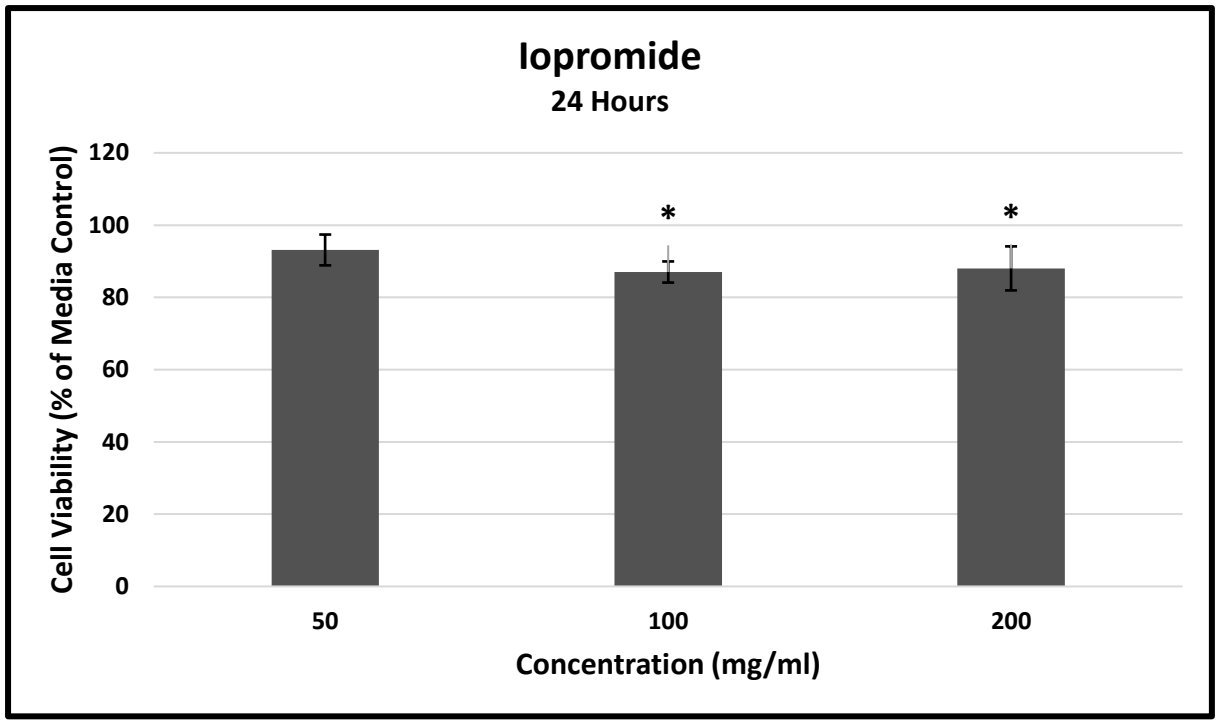

Figure 3.14 Bar graph illustrating the effect of treatment with varying concentrations of Iopromide for 24 hours on cell viability of primary human chondrocytes using the MTS assay. Data represents six independent experiments

*Statistically significant difference in cell viability from media control

3.4.2 Time-dependent effect of Iopromide on primary human chondrocytes

3.4.2.1 Iopromide: 50 mg/ml

A Shapiro-Wilks test and Kolmogorov-Smirnov test ($p > 0.05$) and a visual inspection of their histograms, normal Q-Q plots and box plots showed that cell viability scores were approximately normally distributed for each time point, with a skewness of -0.38 (SE = 0.91) and a kurtosis of -1.5 (SE = 2.0) for 1 hour, a skewness of 1.39 (SE = 0.91) and a kurtosis of 1.4 (SE = 2.0) for 4 hours, a skewness of 0.74 (SE = 0.91) and a kurtosis of 2.19 (SE = 2.0) for 16 hours and a skewness of 1.23 (SE = 1.01) and a kurtosis of 0.95 (SE = 2.62) for 24 hours.

There was no significant time-dependent effect of Iopromide 50 mg/ml on cell viability ([1 hour – 4 hours, $p = 0.66$; 95% CI, -3.3 – 11.7], [1 hour – 16 hours, $p = 1.0$; 95% CI, -6.7 – 8.4], [1 hour – 24 hours, $p = 1.0$; 95% CI, -6.4 – 9.5]), [4 hours – 16 hours, $p = 0.36$; 95% CI, -2.5 – 12.6]), [4 hours – 24 hours, $p = 0.26$; 95% CI, -2.2 – 13.7]) and [16 hours – 24 hours, $p = 1.0$; 95% CI, -7.2 – 8.7]).

Table 3.15 Effect of treatment with Iopromide 50 mg/ml for varying time periods on cell viability of primary human chondrocytes using the MTS assay. Data represents six independent experiments

	1 Hour	**4 Hours**	**16 Hours**	**24 Hours**	***p*-value**
Mean	94.7	99.5	93.9	93.1	
95% CI	92.4 – 99.9	97.5 – 101.5	86.3 – 101.4	86.3 – 90.7	
SD	1.8	1.9	6.1	4.3	
Range	92.2 – 96.5	97.5 – 101.6	86.3 – 103.3	89.5 – 99.1	
					$p = 0.14$

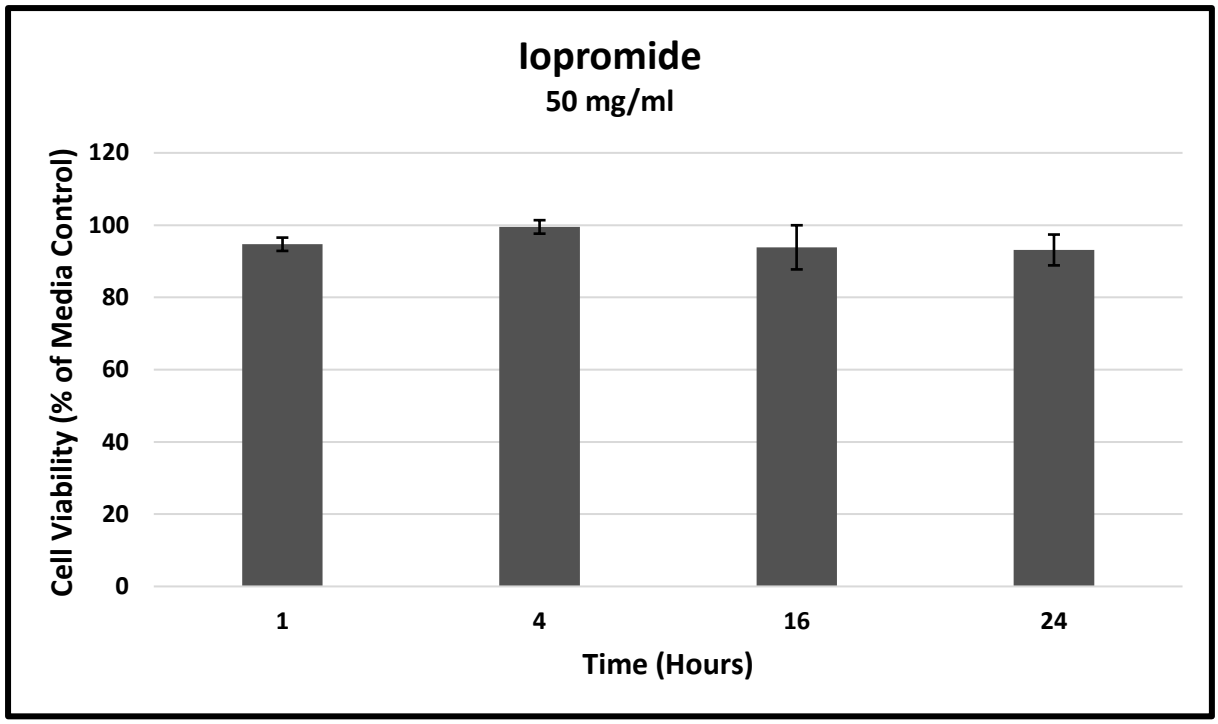

Figure 3.15 Bar graph illustrating the effect of treatment with Iopromide 50 mg/ml for varying time periods on cell viability of primary human chondrocytes using the MTS assay. Data represents six independent experiments

3.4.2.2 Iopromide: 100 mg/ml

A Shapiro-Wilks test and Kolmogorov-Smirnov test ($p > 0.05$) and a visual inspection of their histograms, normal Q-Q plots and box plots showed that cell viability scores were approximately normally distributed for each time point, with a skewness of -0.5 (SE = 0.91) and a kurtosis of -1.1 (SE = 2.0) for 1 hour, a skewness of -0.04 (SE = 0.91) and a kurtosis of 0.75 (SE = 2.0) for 4 hours, a skewness of 0.54 (SE = 0.91) and a kurtosis of 0.67 (SE = 2.0) for 16 hours and a skewness of 1.7 (SE = 0.91) and a kurtosis of 3.1 (SE = 2.0) for 24 hours.

There was a significant time-dependent effect of Iopromide 100 mg/ml on cell viability between 1 hour and 24 hours ($p = 0.02$; 95% CI, 1.0 – 17.1) and between 4 hours and 24 hours ($p < 0.05$; 95% CI, 0.05 – 16.2). There was no significant difference between 1 hour and 4 hours ($p = 1.0$; 95% CI, -7.1 – 9.0), 1 hour and 16 hours ($p = 0.72$; 95% CI, -3.6 – 12.4), 4 hours and 16 hours ($p = 1.0$; 95% CI, -4.6 – 11.5) and 16 hours and 24 hours ($p = 0.6$; 95% CI, -3.4 – 12.7).

Table 3.16 Effect of treatment with Iopromide 100 mg/ml for varying time periods on cell viability of primary human chondrocytes using the MTS assay. Data represents six independent experiments

	1 Hour	**4 Hours**	**16 Hours**	**24 Hours**	***p*-value**
Mean	96.1	94.8	91.7	87.0	
95% CI	89.9 – 102.3	92.0 – 97.7	84.5 – 98.9	83.4 – 90.7	
SD	4.9	2.7	5.8	2.9	
Range	89.3 – 101.8	92.1 – 98.1	84.7 – 100.2	84.8 – 92.0	
					$p = 0.02$

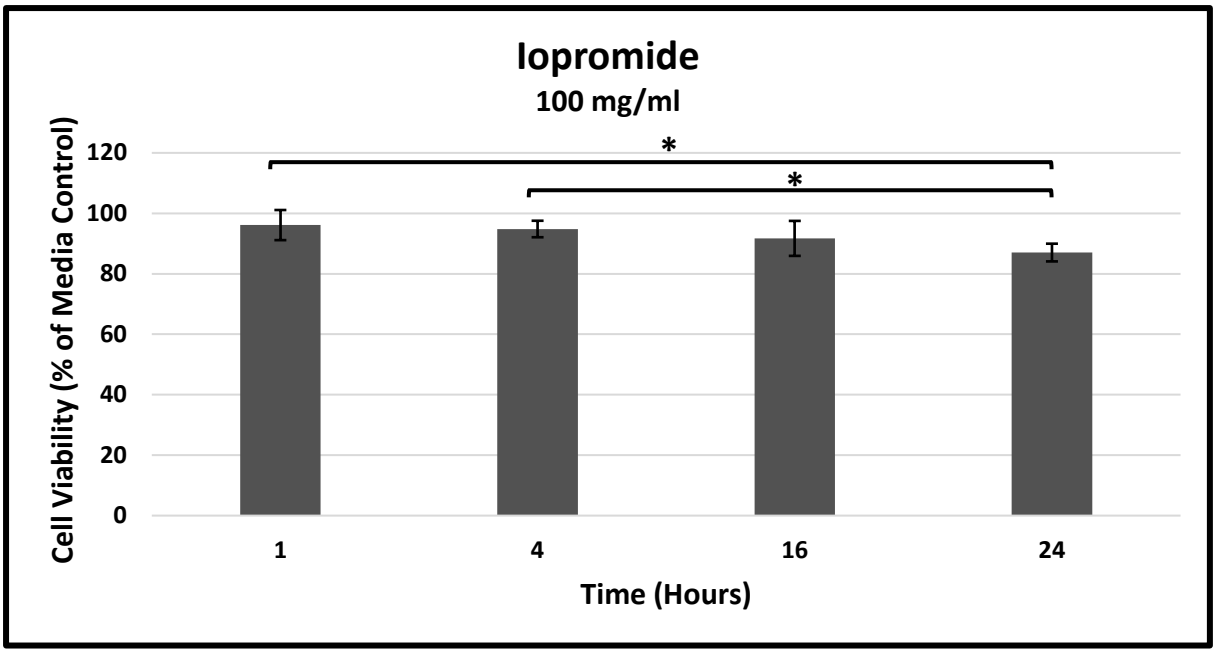

Figure 3.16 Bar graph illustrating the effect of treatment with Iopromide 100 mg/ml for varying time periods on cell viability of primary human chondrocytes using the MTS assay. Data represents six independent experiments

* Statistically significant difference in cell viability between time periods

3.4.2.3 Iopromide: 200 mg/ml

A Shapiro-Wilks test and Kolmogorov-Smirnov test ($p > 0.05$) and a visual inspection of their histograms, normal Q-Q plots and box plots showed that cell viability scores were approximately normally distributed for each time point, with a skewness of 0.90 (SE = 0.91) and a kurtosis of 1.57 (SE = 2.0) for 1 hour, a skewness of -0.04 (SE = 0.69) and a kurtosis of -2.04 (SE = 1.33) for 4 hours, a skewness of 0.57 (SE = 0.91) and a kurtosis of 0.13 (SE = 2.0) for 16 hours and a skewness of -0.27 (SE = 0.91) and a kurtosis of -3.0 (SE = 2.0) for 24 hours.

There was no significant time-dependent effect of Iopromide 200 mg/ml on cell viability ([1 hour – 4 hours, $p = 1.0$; 95% CI, -8.5 – 14.4], [1 hour – 16 hours, $p = 1.0$; 95% CI, -8.4 – 18.1], [1 hour – 24 hours, $p = 0.69$; 95% CI, -5.8 – 20.7]), [4 hours – 16 hours, $p = 1.0$; 95% CI, -9.5 – 13.4]), [4 hours – 24 hours, $p = 1.0$; 95% CI, -6.9 – 16.0]) and [16 hours – 24 hours, $p = 1.0$; 95% CI, -10.6 – 15.9]).

Table 3.17 Effect of treatment with Iopromide 200 mg/ml for varying time periods on cell viability of primary human chondrocytes using the MTS assay. Data represents six independent experiments

	1 Hour	**4 Hours**	**16 Hours**	**24 Hours**	***p*-value**
Mean	95.5	92.6	90.7	88.0	
95% CI	80.3 – 110.7	88.4 – 96.7	87.0 – 94.3	80.5 – 95.6	
SD	12.2	5.8	2.9	6.1	
Range	81.5 – 114.6	84.7 – 99.7	87.4 – 94.9	81.6 – 94.2	
					$p = 0.42$

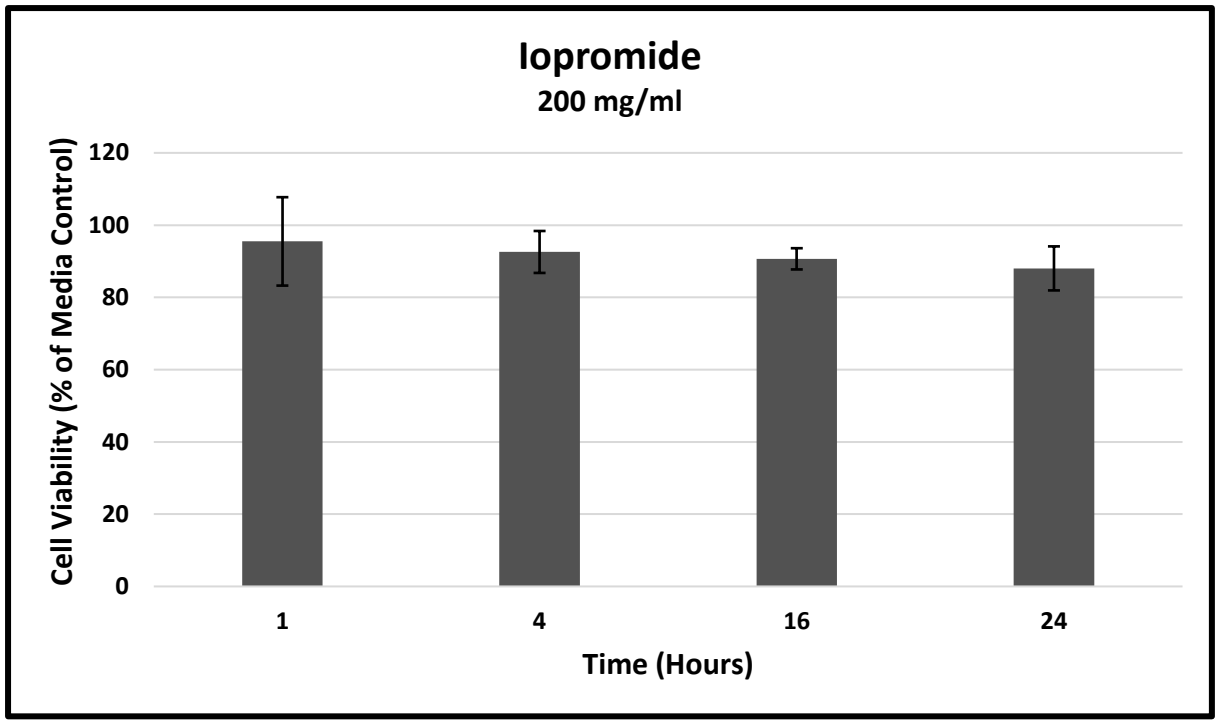

Figure 3.17 Bar graph illustrating the effect of treatment with Iopromide 200 mg/ml for varying time periods on cell viability of primary human chondrocytes using the MTS assay. Data represents six independent experiments

3.5 The effect of Gadopentetate Dimeglumine on primary human chondrocytes

To investigate the concentration-dependent and time-dependent effect of Gadopentetate Dimeglumine on primary human chondrocyte cell viability, cells were treated with varying concentrations of Gadopentetate Dimeglumine (either 1.7 mmol/L, 2.5 mmol/L, 5 mmol/L or 10 mmol/L) for 1 hour, 4 hours, 16 hours and 24 hours (Section 2.3.5.2.1). Cells exposed to cell culture media served as the controls. Cell viability was then assessed using the MTS assay (Section 2.3.6.1). All data represents the results of six independent experiments.

3.5.1 Concentration-dependent effect Gadopentetate Dimeglumine on primary human chondrocytes

3.5.1.1 Gadopentetate Dimeglumine: 1 Hour

A Shapiro-Wilks test and Kolmogorov-Smirnov test ($p > 0.05$) and a visual inspection of their histograms, normal Q-Q plots and box plots showed that cell viability scores were approximately normally distributed for each concentration, with a skewness of -0.23 (SE = 0.52) and a kurtosis of 0.13 (SE = 1.01) for 1.7 mmol/L , a skewness of 0.32 (SE = 0.47) and a kurtosis of 0.66 (SE = 0.92) for 2.5 mmol/L, a skewness of 0.65 (SE = 0.47) and a kurtosis of 2.3 (SE = 0.92) for 5 mmol/L and a skewness of -0.24 (SE = 0.52) and a kurtosis of 0.14 (SE = 1.02) for 10 mmol/L.

At 1 hour there was no significant difference in cell viability between the media control and a Gadopentetate Dimeglumine concentration of either 1.7 mmol/L ($p = 1.0$; 95% CI, -11.9 – 13.6), 2.5 mmol/L ($p = 1.0$; 95% CI, -10.7 – 13.4), 5 mmol/L ($p = 1.0$; 95% CI, -8.5 – 15.6) or 10 mmol/L ($p = 1.0$; 95% CI, -10.4 – 15.2).

There was no significant concentration-dependent effect of Gadopentetate Dimeglumine on cell viability ([1.7 – 2.5 mmol/L, $p = 1.0$; 95% CI, -12.2 – 13.3], [1.7 – 5 mmol/L, $p = 1.0$; 95% CI, -8.5 – 17.1], [1.7 – 10 mmol/L, $p = 1.0$; 95% CI, -11.9 – 15.2], [2.5 – 5 mmol/L, $p = 1.0$; 95% CI, -4.2 – 16.9], [2.5 – 10 mmol/L, $p = 1.0$; 95% CI, -11.7 – 13.9], [5 – 10 mmol/L, $p = 1.0$; 95% CI, -0.8 – 17.2]).

Table 3.18 Effect of treatment with varying concentrations of Gadopentetate Dimeglumine for 1 hour on cell viability of primary human chondrocytes using the MTS assay. Data represents six independent experiments

	1.7 mmol/L	2.5 mmol/L	5 mmol/L	10 mmol/L	*p*-value
Mean	99.0	99.8	98.0	97.6	
95% CI	88.9 – 112.1	86.3 – 113.2	93.5 – 102.4	93.0 – 102.3	
SD	8.2	10.8	3.6	1.9	
Range	87.1 – 105.9	89.2 – 115.8	95.1 – 103.3	96.5 – 99.8	
					$p = 0.7$

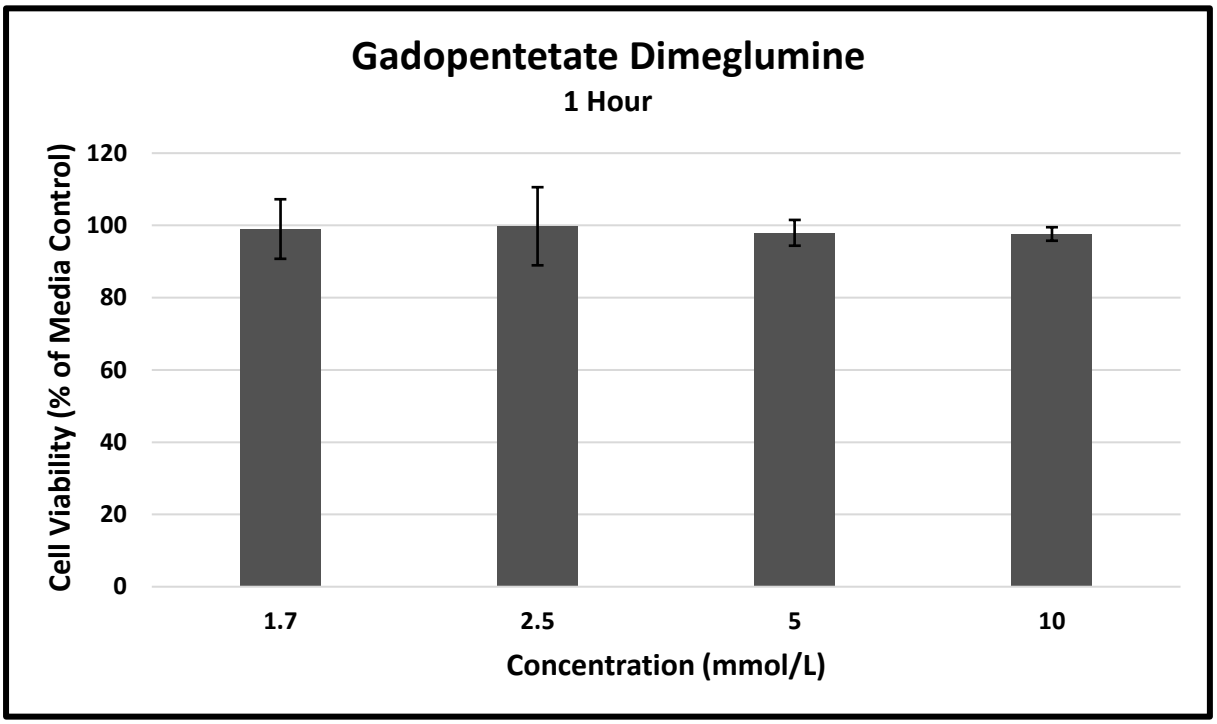

Figure 3.18 Bar graph illustrating the effect of treatment with varying concentrations of Gadopentetate Dimeglumine for 1 hour on cell viability of primary human chondrocytes using the MTS assay. Data represents six independent experiments

3.5.1.2 Gadopentetate Dimeglumine: 4 Hours

A Shapiro-Wilks test and Kolmogorov-Smirnov test ($p > 0.05$) and a visual inspection of their histograms, normal Q-Q plots and box plots showed that cell viability scores were approximately normally distributed for each concentration, with a skewness of 0.87 (SE = 0.58) and a kurtosis of 0.96 (SE = 1.12) for 1.7 mmol/L , a skewness of 0.03 (SE = 0.51) and a kurtosis of 1.5 (SE = 1.0) for 2.5 mmol/L, a skewness of 1.0 (SE = 0.51) and a kurtosis of 1.6 (SE = 1.0) for 5 mmol/L and a skewness of 0.63 (SE = 0.58) and a kurtosis of -0.61 (SE = 1.12) for 10 mmol/L.

At 1 hour there was no significant difference in cell viability between the media control and a Gadopentetate Dimeglumine concentration of either 1.7 mmol/L ($p = 1.0$; 95% CI, -9.2 – 9.9), 2.5 mmol/L ($p = 1.0$; 95% CI, -8.6 – 8.9), 5 mmol/L ($p = 1.0$; 95% CI, -7.9 – 9.8) or 10 mmol/L ($p = 1.0$; 95% CI, -8.0 – 11.0).

There was no significant concentration-dependent effect of Gadopentetate Dimeglumine on cell viability ([1.7 – 2.5 mmol/L, $p = 1.0$; 95% CI, -9.0 – 10.0], [1.7 – 5 mmol/L, $p = 1.0$; 95% CI, -8.2 – 10.8], [1.7 – 10 mmol/L, $p = 1.0$; 95% CI, -8.3 – 12.0], [2.5 – 5 mmol/L, $p = 1.0$; 95% CI, -8.0 – 9.6], [2.5 – 10 mmol/L, $p = 1.0$; 95% CI, -8.2 – 10.9], [5 – 10 mmol/L, $p = 1.0$; 95% CI, -8.9 – 10.1]).

Table 3.19 Effect of treatment with varying concentrations of Gadopentetate Dimeglumine for 4 hours on cell viability of primary human chondrocytes using the MTS assay. Data represents six independent experiments

	1.7 mmol/L	**2.5 mmol/L**	**5 mmol/L**	**10 mmol/L**	***p*-value**
Mean	98.8	98.4	97.3	98.2	
95% CI	90.6 – 107.1	93.4 – 103.4	87.0 – 107.6	90.7 – 105.6	
SD	3.3	3.1	6.5	3.0	
Range	95.3 – 101.9	95.5 – 102.6	88.2 – 102.3	96.6 – 101.6	
					$p = 0.98$

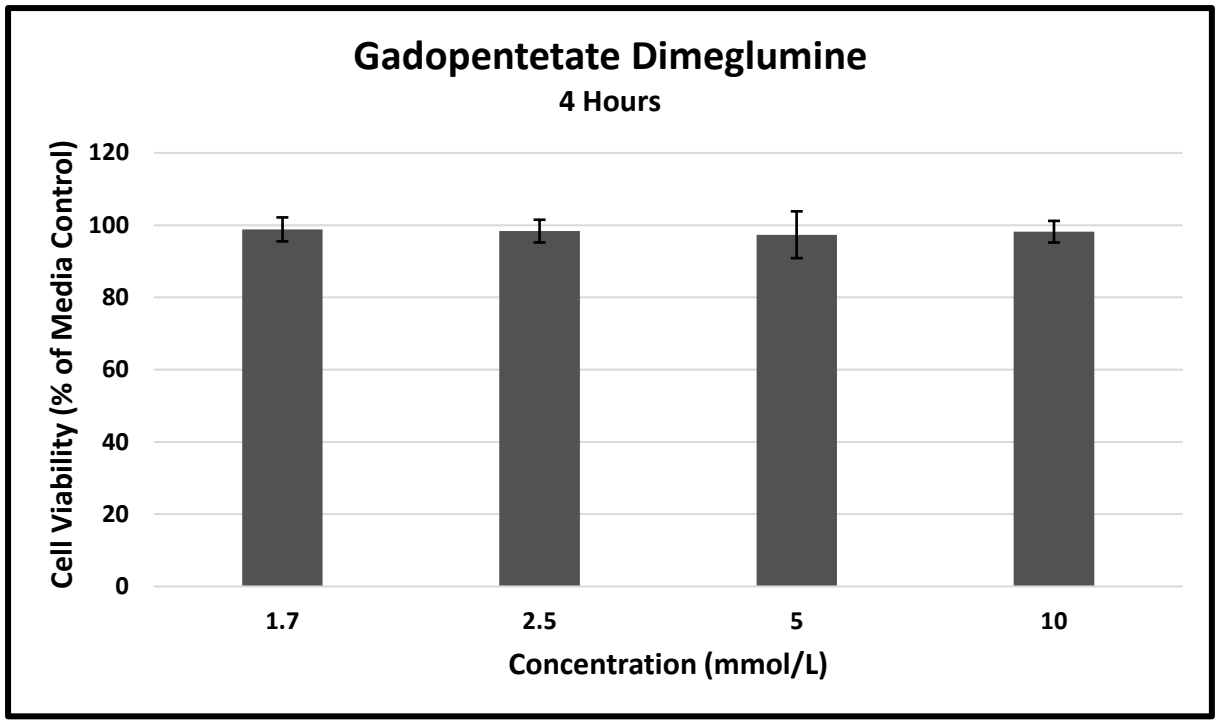

Figure 3.19 Bar graph illustrating the effect of treatment with varying concentrations of Gadopentetate Dimeglumine for 4 hours on cell viability of primary human chondrocytes using the MTS assay. Data represents six independent experiments

3.5.1.3 Gadopentetate Dimeglumine: 16 Hours

A Shapiro-Wilks test and Kolmogorov-Smirnov test ($p > 0.05$) and a visual inspection of their histograms, normal Q-Q plots and box plots showed that cell viability scores were approximately normally distributed for each concentration, with a skewness of 0.31 (SE = 0.85) and a kurtosis of -1.9 (SE = 1.7) for 1.7 mmol/L , a skewness of -1.23 (SE = 0.85) and a kurtosis of 1.34 (SE = 1.7) for 2.5 mmol/L, a skewness of -0.2 (SE = 0.79) and a kurtosis of -0.44 (SE = 1.59) for 5 mmol/L and a skewness of 1.03 (SE = 0.79) and a kurtosis of 2.15 (SE = 1.59) for 10 mmol/L.

At 1 hour there was no significant difference in cell viability between the media control and a Gadopentetate Dimeglumine concentration of either 1.7 mmol/L ($p = 1.0$; 95% CI, -12.1 – 14.6), 2.5 mmol/L ($p = 1.0$; 95% CI, -11.7 – 15.1), 5 mmol/L ($p = 1.0$; 95% CI, -10.3 – 15.4) or 10 mmol/L ($p = 1.0$; 95% CI, -11.5 – 14.2).

There was no significant concentration-dependent effect of Gadopentetate Dimeglumine on cell viability ([1.7 – 2.5 mmol/L, $p = 1.0$; 95% CI, -13.4 – 14.3], [1.7 – 5 mmol/L, $p = 1.0$; 95% CI, -14.7 – 21.1], [1.7 – 10 mmol/L, $p = 1.0$; 95% CI, -13.3 – 13.4], [2.5 – 5 mmol/L, $p = 1.0$; 95% CI, -12.6 – 14.2], [2.5 – 10 mmol/L, $p = 1.0$; 95% CI, -13.0 – 13.8], [5 – 10 mmol/L, $p = 1.0$; 95% CI, -11.6 – 14.1]).

Table 3.20 Effect of treatment with varying concentrations of Gadopentetate Dimeglumine for 16 hours on cell viability of primary human chondrocytes using the MTS assay. Data represents six independent experiments

	1.7 mmol/L	**2.5 mmol/L**	**5 mmol/L**	**10 mmol/L**	***p*-value**
Mean	98.8	98.3	97.5	98.7	
95% CI	93.6 – 103.9	88.6 – 107.7	88.5 – 106.2	88.8 – 108.6	
SD	4.9	9.0	9.4	10.7	
Range	93.7 – 105.0	82.5 – 106.7	82.9 – 110.4	84.8 – 119.0	
					$p = 0.98$

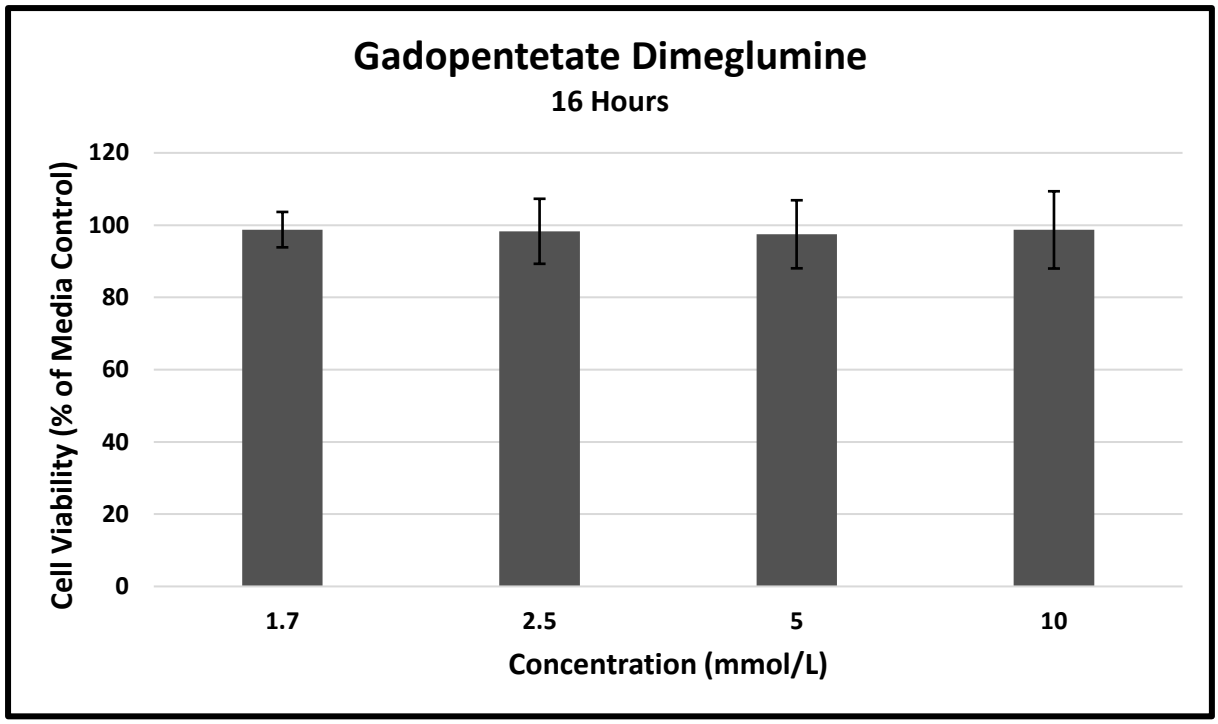

Figure 3.20 Bar graph illustrating the effect of treatment with varying concentrations of Gadopentetate Dimeglumine for 16 hours on cell viability of primary human chondrocytes using the MTS assay. Data represents six independent experiments

3.5.1.4 Gadopentetate Dimeglumine: 24 Hours

A Shapiro-Wilks test and Kolmogorov-Smirnov test ($p > 0.05$) and a visual inspection of their histograms, normal Q-Q plots and box plots showed that cell viability scores were approximately normally distributed for each concentration, with a skewness of -0.12 (SE = 0.91) and a kurtosis of -1.99 (SE = 2.0) for 1.7 mmol/L , a skewness of 0.55 (SE = 0.85) and a kurtosis of -1.7 (SE = 1.7) for 2.5 mmol/L, a skewness of 0.47 (SE = 0.85) and a kurtosis of -1.74 (SE = 1.7) for 5 mmol/L and a skewness of 0.40 (SE = 0.91) and a kurtosis of -2.01 (SE = 2.0) for 10 mmol/L.

At 1 hour there was no significant difference in cell viability between the media control and a Gadopentetate Dimeglumine concentration of either 1.7 mmol/L (p = 1.0; 95% CI, -7.0 – 16.6), 2.5 mmol/L (p = 1.0; 95% CI, -16.0 – 16.4), 5 mmol/L (p = 1.0; 95% CI, -9.5 – 12.9) or 10 mmol/L (p = 1.0; 95% CI, -10.6 – 12.9).

There was no significant concentration-dependent effect of Gadopentetate Dimeglumine on cell viability ([1.7 – 2.5 mmol/L, p = 1.0; 95% CI, -11.4 – 12.2], [1.7 – 5 mmol/L, p = 1.0; 95% CI, -8.7 – 14.9], [1.7 – 10 mmol/L, p = 1.0; 95% CI, -8.7 – 15.9], [2.5 – 5 mmol/L, p = 1.0; 95% CI, -7.7 – 14.7], [2.5 – 10 mmol/L, p = 1.0; 95% CI, -7.8 – 15.8], [5 – 10 mmol/L, p = 1.0; 95% CI, -11.3 – 12.3]).

Table 3.21 Effect of treatment with varying concentrations of Gadopentetate Dimeglumine for 24 hours on cell viability of primary human chondrocytes using the MTS assay. Data represents six independent experiments

	1.7 mmol/L	**2.5 mmol/L**	**5 mmol/L**	**10 mmol/L**	***p*-value**
Mean	95.2	94.8	98.3	98.8	
95% CI	87.3 – 103.0	88.3 – 101.3	92.6 – 103.9	86.3 – 111.4	
SD	6.3	6.2	5.4	10.1	
Range	87.1 – 101.7	87.9 – 102.7	93.5 – 106.2	88.1 – 111.9	
					p = 0.56

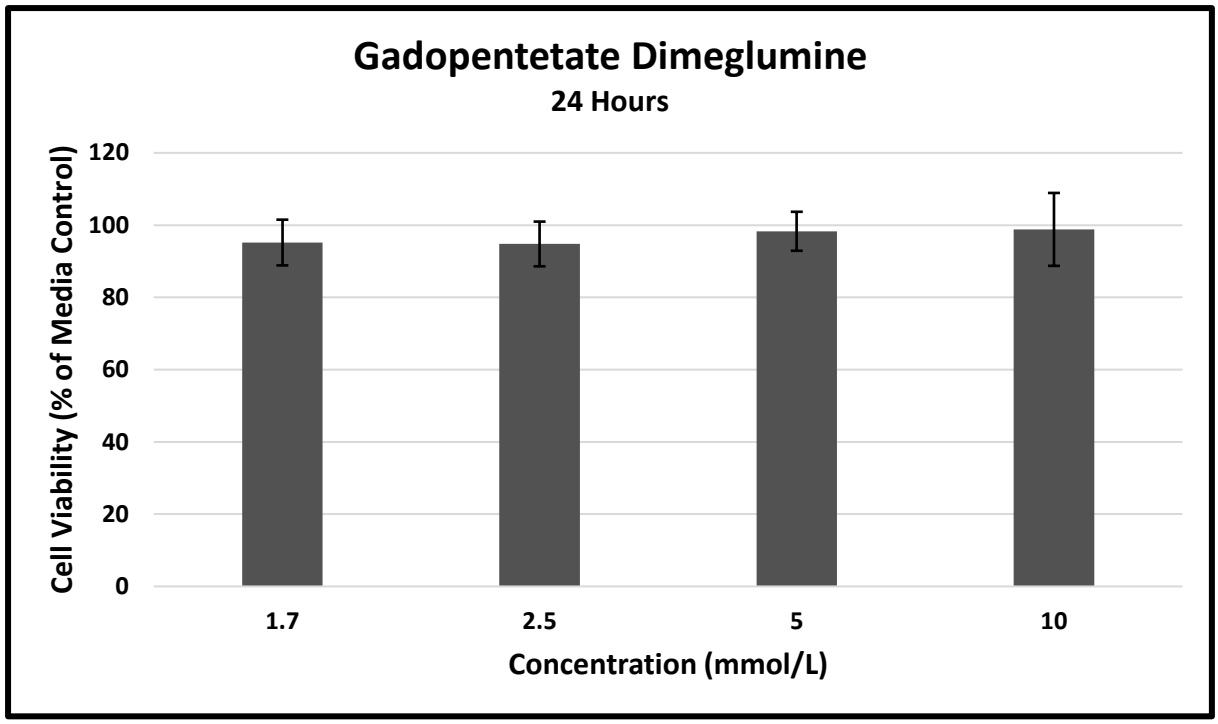

Figure 3.21 Bar graph illustrating the effect of treatment with varying concentrations of Gadopentetate Dimeglumine for 24 hours on cell viability of primary human chondrocytes using the MTS assay. Data represents six independent experiments

3.5.2 Time-dependent effect Gadopentetate Dimeglumine on primary human chondrocytes

3.5.2.1 Gadopentetate Dimeglumine: 1.7 mmol/L (1:300 v:v)

A Shapiro-Wilks test and Kolmogorov-Smirnov test ($p > 0.05$) and a visual inspection of their histograms, normal Q-Q plots and box plots showed that cell viability scores were approximately normally distributed for each time point, with a skewness of -1.6 (SE = 1.01) and a kurtosis of 2.9 (SE = 2.6) for 1 hour, a skewness of -0.68 (SE = 1.23) and a kurtosis of 0.0 (SE = 0.0) for 4 hours, a skewness of 0.31 (SE = 0.85) and a kurtosis of -1.93 (SE = 1.74) for 16 hours and a skewness of -0.12 (SE = 0.91) and a kurtosis of -1.9 (SE = 2.0) for 24 hours.

There was no significant time-dependent effect of Gadopentetate Dimeglumine 1.7 mmol/L on cell viability ([1 hour – 4 hours, $p = 1.0$; 95% CI, -13.9 – 14.3], [1 hour – 16 hours, $p = 1.0$; 95% CI, -11.7 – 12.2], [1 hour – 24 hours, $p = 1.0$; 95% CI, -8.6 – 16.2]), [4 hours – 16 hours, $p = 1.0$; 95% CI, -12.9 – 13.1]), [4 hours – 24 hours, $p = 1.0$; 95% CI, -9.8 – 17.1]) and [16 hours – 24 hours, $p = 1.0$; 95% CI, -7.6 – 14.7]).

Table 3.22 Effect of treatment with Gadopentetate Dimeglumine (1.7 mmol/L; 1:300 *v:v*) for varying time periods on cell viability of primary human chondrocytes using the MTS assay. Data represents six independent experiments.

	1 Hour	**4 Hours**	**16 Hours**	**24 Hours**	***p*-value**
Mean	99.0	98.8	98.8	95.2	
95% CI	88.9 – 112.1	90.6 – 107.1	93.6 – 103.9	87.3 – 103.0	
SD	8.2	3.3	4.9	6.3	
Range	87.1 – 105.9	95.3 – 101.9	93.7 – 105.0	87.1 – 101.7	
					$p = 0.72$

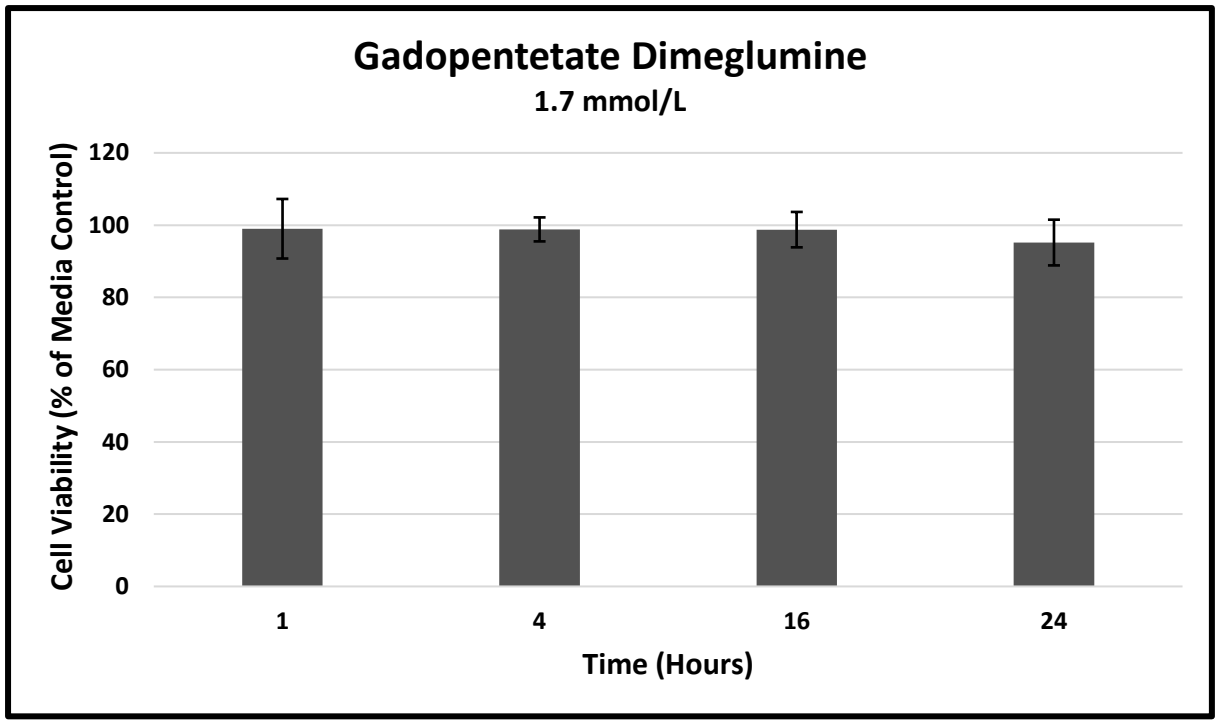

Figure 3.22 Bar graph illustrating the effect of treatment with Gadopentetate Dimeglumine (1.7 mmol/L; 1:300 *v:v*) for varying time periods on cell viability of primary human chondrocytes using the MTS assay. Data represents six independent experiments.

3.5.2.2 Gadopentetate Dimeglumine: 2.5 mmol/L (1:200)

A Shapiro-Wilks test and Kolmogorov-Smirnov test ($p > 0.05$) and a visual inspection of their histograms, normal Q-Q plots and box plots showed that cell viability scores were approximately normally distributed for each time point, with a skewness of 0.92 (SE = 0.91) and a kurtosis of -0.47 (SE = 2.0) for 1 hour, a skewness of 1.07 (SE = 1.01) and a kurtosis of 0.7 (SE = 2.62) for 4 hours, a skewness of -1.23 (SE = 0.85) and a kurtosis of 1.34 (SE = 1.7) for 16 hours and a skewness of 0.55 (SE = 0.85) and a kurtosis of -1.76 (SE = 1.7) for 24 hours.

There was no significant time-dependent effect of Gadopentetate Dimeglumine 2.5 mmol/L on cell viability ([1 hour – 4 hours, $p = 1.0$; 95% CI, -14.6 – 17.5], [1 hour – 16 hours, $p = 1.0$; 95% CI, -13.0 – 15.9], [1 hour – 24 hours, $p = 1.0$; 95% CI, -9.5 – 19.5]), [4 hours – 16 hours, $p = 1.0$; 95% CI, -15.3 – 15.5]), [4 hours – 24 hours, $p = 1.0$; 95% CI, -11.9 – 19.0]) and [16 hours – 24 hours, $p = 1.0$; 95% CI, -10.3 – 17.3]).

Table 3.23 Effect of treatment with Gadopentetate Dimeglumine (2.5 mmol/L; 1:200 *v:v*) for varying time periods on cell viability of primary human chondrocytes using the MTS assay. Data represents six independent experiments.

	1 Hour	**4 Hours**	**16 Hours**	**24 Hours**	***p*-value**
Mean	99.8	98.4	98.3	94.8	
95% CI	86.3 – 113.2	93.4 – 103.4	88.6 – 107.7	88.3 – 101.3	
SD	10.8	3.1	9.0	6.2	
Range	89.2 – 115.8	95.5 – 102.6	82.5 – 106.7	87.9 – 102.7	
					$p = 0.76$

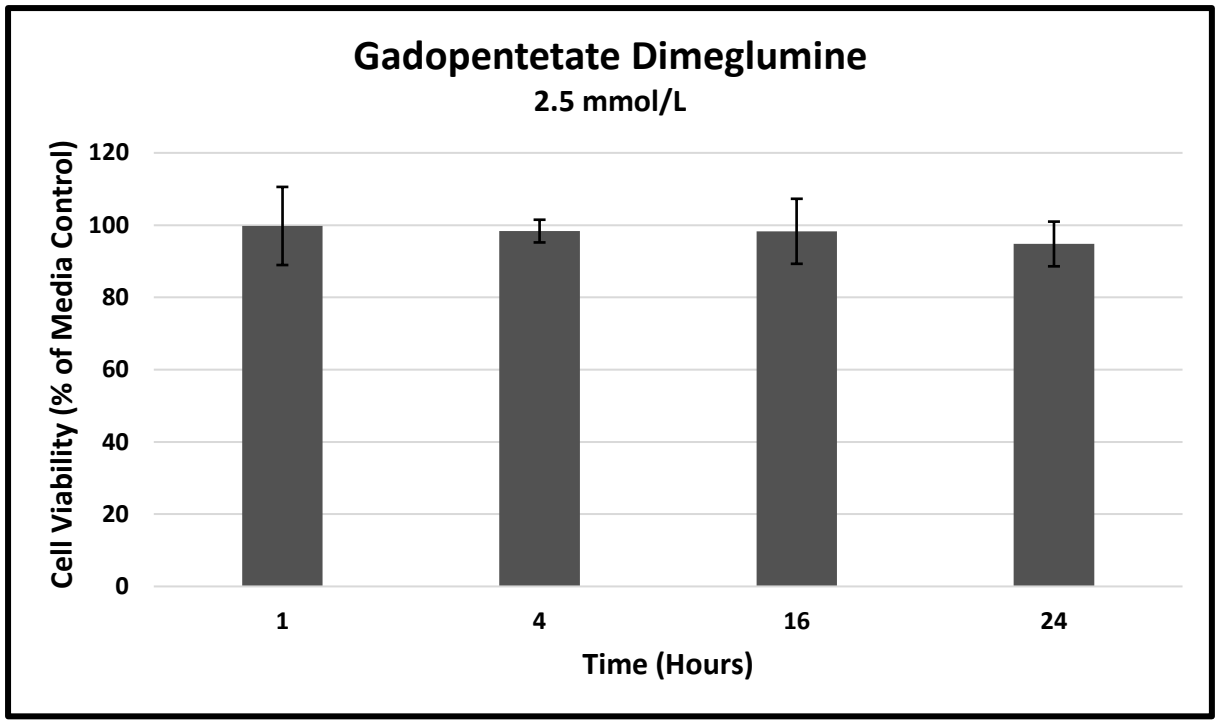

Figure 3.23 Bar graph illustrating the effect of treatment with Gadopentetate Dimeglumine (2.5 mmol/L; 1:200 *v:v*) for varying time periods on cell viability of primary human chondrocytes using the MTS assay. Data represents six independent experiments.

3.5.2.3 Gadopentetate Dimeglumine: 5 mmol/L (1:100)

A Shapiro-Wilks test and Kolmogorov-Smirnov test ($p > 0.05$) and a visual inspection of their histograms, normal Q-Q plots and box plots showed that cell viability scores were approximately normally distributed for each time point, with a skewness of 1.04 (SE = 0.91) and a kurtosis of -0.57 (SE = 2.0) for 1 hour, a skewness of -1.39 (SE = 1.01) and a kurtosis of 1.48 (SE = 2.62) for 4 hours, a skewness of -0.20 (SE = 0.79) and a kurtosis of -0.44 (SE = 1.59) for 16 hours and a skewness of 0.47 (SE = 0.85) and a kurtosis of -1.74 (SE = 1.74) for 24 hours.

There was no significant time-dependent effect of Gadopentetate Dimeglumine 5 mmol/L on cell viability ([1 hour – 4 hours, $p = 1.0$; 95% CI, -13.1 – 14.3], [1 hour – 16 hours, $p = 1.0$; 95% CI, -11.5 – 12.4], [1 hour – 24 hours, $p = 1.0$; 95% CI, -12.0 – 12.7]), [4 hours – 16 hours, $p = 1.0$; 95% CI, -12.2 – 14.1]), [4 hours – 24 hours, $p = 1.0$; 95% CI, -12.2 – 14.1]) and [16 hours – 24 hours, $p = 1.0$; 95% CI, -7.5 – 12.2]).

Table 3.24 Effect of treatment with Gadopentetate Dimeglumine (5 mmol/L; 1:100 *v:v*) for varying time periods on cell viability of primary human chondrocytes using the MTS assay. Data represents six independent experiments

	1 Hour	**4 Hours**	**16 Hours**	**24 Hours**	***p*-value**
Mean	98.0	97.3	97.5	98.3	
95% CI	93.5 – 102.4	87.0 – 107.6	88.5 – 106.2	92.6 – 103.9	
SD	3.6	6.5	9.4	5.4	
Range	95.1 – 103.3	88.2 – 102.3	82.9 – 110.4	93.5 – 106.2	
					$p = 0.995$

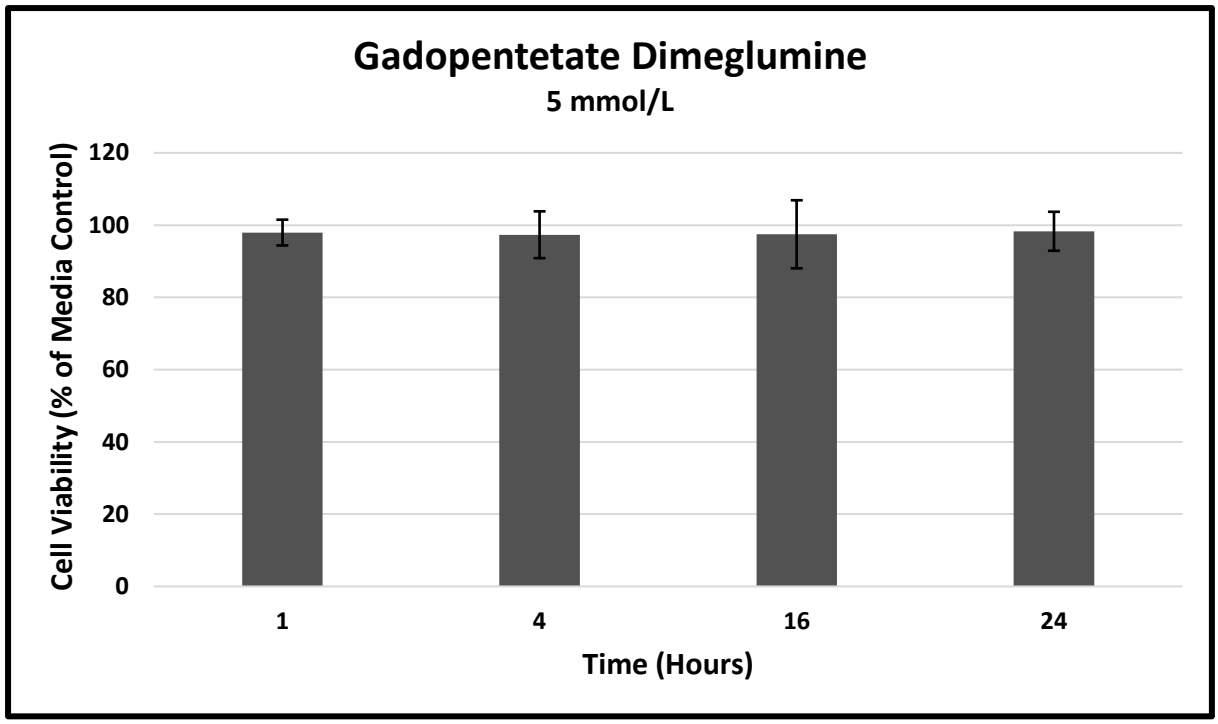

Figure 3.24 Bar graph illustrating the effect of treatment with Gadopentetate Dimeglumine (5 mmol/L; 1:100 *v:v*) for varying time periods on cell viability of primary human chondrocytes using the MTS assay. Data represents six independent experiments.

3.5.2.4 Gadopentetate Dimeglumine: 10 mmol/L (1:50)

A Shapiro-Wilks test and Kolmogorov-Smirnov test ($p > 0.05$) and a visual inspection of their histograms, normal Q-Q plots and box plots showed that cell viability scores were approximately normally distributed for each time point, with a skewness of 0.66 (SE = 0.41) and a kurtosis of 1.17 (SE = 0.79) for 1 hour, a skewness of 0.63 (SE = 0.58) and a kurtosis of -0.61 (SE = 1.12) for 4 hours, a skewness of 0.23 (SE = 0.40) and a kurtosis of 1.1 (SE = 0.78) for 16 hours and a skewness of 0.7 (SE = 0.41) and a kurtosis of 1.16 (SE = 0.8) for 24 hours.

There was no significant time-dependent effect of Gadopentetate Dimeglumine 10 mmol/L on cell viability ([1 hour – 4 hours, $p = 1.0$; 95% CI, -11.8 – 19.7], [1 hour – 16 hours, $p = 1.0$; 95% CI, -10.2 – 15.9], [1 hour – 24 hours, $p = 1.0$; 95% CI, -5.8 – 20.5]), [4 hours – 16 hours, $p = 1.0$; 95% CI, -13.0 – 15.2]), [4 hours – 24 hours, $p = 1.0$; 95% CI, -10.8 – 17.7]) and [16 hours – 24 hours, $p = 1.0$; 95% CI, -6.6 – 15.6]).

Table 3.25 Effect of treatment with Gadopentetate Dimeglumine (10 mmol/L; 1:50 *v:v*) for varying time periods on cell viability of primary human chondrocytes using the MTS assay. Data represents six independent experiments

	1 Hour	**4 Hours**	**16 Hours**	**24 Hours**	***p*-value**
Mean	97.6	98.2	98.7	98.8	
95% CI	93.0 – 102.3	90.7 – 105.6	88.8 – 108.6	86.3 – 111.4	
SD	1.9	3.0	10.7	10.1	
Range	96.5 – 99.8	96.6 – 101.6	84.8 – 119.0	88.1 – 111.9	
					$p = 0.48$

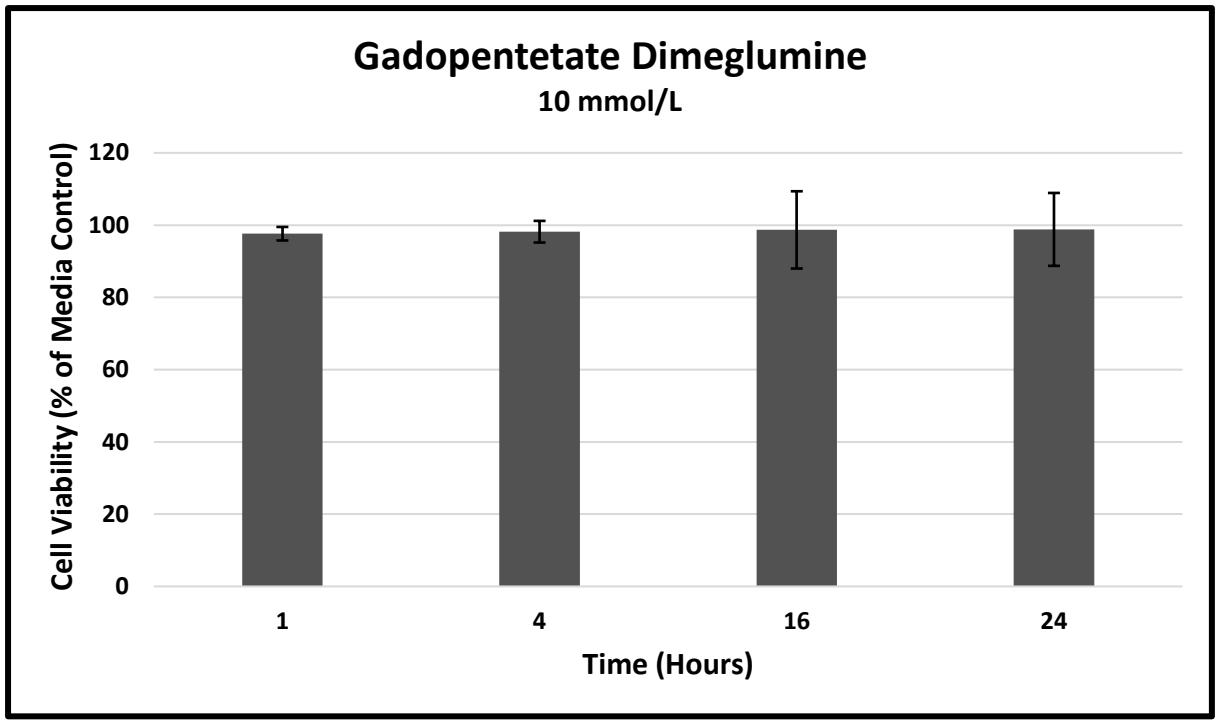

Figure 3.25 Bar graph illustrating the effect of treatment with Gadopentetate Dimeglumine (10 mmol/L; 1:50 *v:v*) for varying time periods on cell viability of primary human chondrocytes using the MTS assay. Data represents six independent experiments.

3.6 The effect of Gadobenate Dimeglumine on primary human chondrocytes

To investigate the concentration-dependent and time-dependent effect of Gadobenate Dimeglumine on primary human chondrocyte cell viability, cells were treated with varying concentrations of Gadobenate Dimeglumine (either 1.7 mmol/L, 2.5 mmol/L, 5 mmol/L or 10 mmol/L) for 1 hour, 4 hours, 16 hours and 24 hours (Section 2.3.5.2.2). Cells exposed to cell culture media served as the controls. Cell viability was then assessed using the MTS assay (Section 2.3.6.1). All data represents the results of six independent experiments.

3.6.1 Concentration-dependent effect of Gadobenate Dimeglumine on primary human chondrocytes

3.6.1.1 Gadobenate Dimeglumine: 1 Hour

A Shapiro-Wilks test and Kolmogorov-Smirnov test ($p > 0.05$) and a visual inspection of their histograms, normal Q-Q plots and box plots showed that cell viability scores were approximately normally distributed for each concentration, with a skewness of -1.25 (SE = 01.01) and a kurtosis of 1.75 (SE = 2.62) for 1.7 mmol/L , a skewness of -0.98 (SE = 0.91) and a kurtosis of 0.87 (SE = 2.0) for 2.5 mmol/L, a skewness of -0.21 (SE = 0.91) and a kurtosis of -0.82 (SE = 2.0) for 5 mmol/L and a skewness of -0.54 (SE = 0.91) and a kurtosis of -1.11 (SE = 2.0) for 10 mmol/L.

At 1 hour there was no significant difference in cell viability between the media control and a Gadobenate Dimeglumine concentration of either 1.7 mmol/L ($p = 1.0$; 95% CI, -5.6 – 11.5), 2.5 mmol/L ($p = 1.0$; 95% CI, -7.5 – 8.7), 5 mmol/L ($p = 1.0$; 95% CI, -7.1 – 9.1) or 10 mmol/L ($p = 1.0$; 95% CI, -4.5 – 11.6).

There was no significant concentration-dependent effect of Gadobenate Dimeglumine on cell viability ([1.7 – 2.5 mmol/L, $p = 1.0$; 95% CI, -6.2 – 10.9], [1.7 – 5 mmol/L, $p = 1.0$; 95% CI, -6.6 – 10.5], [1.7 – 10 mmol/L, $p = 1.0$; 95% CI, -7.9 - 9.2], [2.5 – 5 mmol/L, $p = 1.0$; 95% CI, -7.7 – 8.4], [2.5 – 10 mmol/L, $p = 1.0$; 95% CI, -5.1 – 11.0], [5 – 10 mmol/L, $p = 1.0$; 95% CI, -5.5 – 10.6]).

Table 3.26 Effect of treatment with varying concentrations of Gadobenate Dimeglumine for 1 hour on cell viability of primary human chondrocytes using the MTS assay. Data represents six independent experiments.

	1.7 mmol/L	2.5 mmol/L	5 mmol/L	10 mmol/L	*p*-value
Mean	97.1	99.4	99.0	96.4	
95% CI	94.0 – 100.1	93.7 – 105.1	92.1 – 105.9	90.7 – 102.2	
SD	1.9	4.6	5.6	4.7	
Range	94.4 – 98.8	92.3 – 104.3	91.5 – 105.8	89.9 – 101.0	
					$p = 0.6$

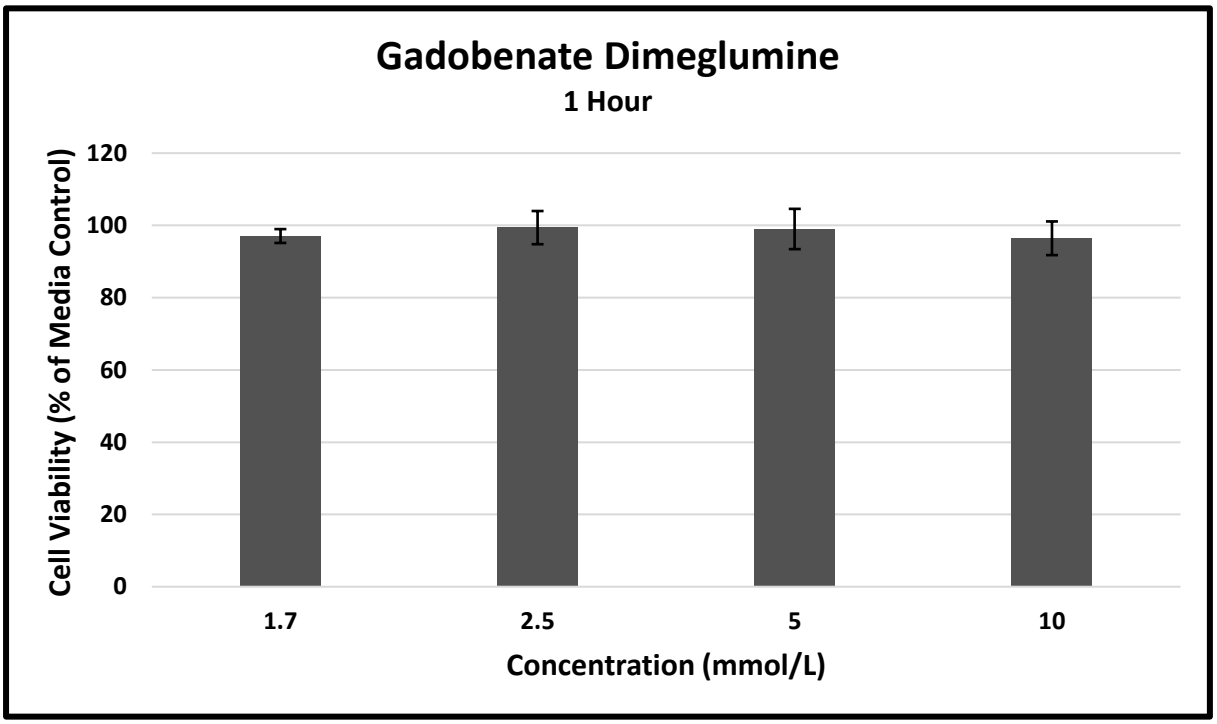

Figure 3.26 Bar graph illustrating the effect of treatment with varying concentrations of Gadobenate Dimeglumine for 1 hour on cell viability of primary human chondrocytes using the MTS assay. Data represents six independent experiments.

3.6.1.2 Gadobenate Dimeglumine: 4 Hours

A Shapiro-Wilks test and Kolmogorov-Smirnov test ($p > 0.05$) and a visual inspection of their histograms, normal Q-Q plots and box plots showed that cell viability scores were approximately normally distributed for each concentration, with a skewness of 1.72 (SE = 1.23) and a kurtosis of 0.1 (SE = 0.12) for 1.7 mmol/L , a skewness of 1.22 (SE = 1.01) and a kurtosis of 2.23 (SE =) f2.62or 2.5 mmol/L, a skewness of 0.08 (SE = 1.01) and a kurtosis of -5.04 (SE = 2.62) for 5 mmol/L and a skewness of 1.84 (SE = 1.01) and a kurtosis of 3.5 (SE = 2.62) for 10 mmol/L.

At 4 hours there was no significant difference in cell viability between the media control and a Gadobenate Dimeglumine concentration of either 1.7 mmol/L (p = 1.0; 95% CI, -10.7 – 12.0), 2.5 mmol/L (p = 0.31; 95% CI, -2.9 – 18.1), 5 mmol/L (p = 1.0; 95% CI, -5.6 – 15.4) or 10 mmol/L (p = 1.0; 95% CI, -8.1 – 12.8).

There was no significant concentration-dependent effect of Gadobenate Dimeglumine on cell viability ([1.7 – 2.5 mmol/L, p = 0.3; 95% CI, -3.1 – 19.6], [1.7 – 5 mmol/L, p = 1.0; 95% CI, -5.8 – 16.9], [1.7 – 10 mmol/L, p = 1.0; 95% CI, -8.3 – 14.4], [2.5 – 5 mmol/L, p = 1.0; 95% CI, -7.9 – 13.2], [2.5 – 10 mmol/L, p = 1.0; 95% CI, -5.3 – 15.7], [5 – 10 mmol/L, p = 1.0; 95% CI, -7.9 – 13.1]).

Table 3.27 Effect of treatment with varying concentrations of Gadobenate Dimeglumine for 4 hours on cell viability of primary human chondrocytes using the MTS assay. Data represents six independent experiments

	1.7 mmol/L	**2.5 mmol/L**	**5 mmol/L**	**10 mmol/L**	***p*-value**
Mean	100.7	92.4	95.1	97.6	
95% CI	86.1 – 115.3	91.1 – 93.8	90.2 – 99.9	85.3 – 109.9	
SD	5.9	0.9	3.1	7.8	
Range	97.1 – 107.4	91.6 – 96.6	92.1 – 98.3	92.2 – 109.1	
					p = 0.12

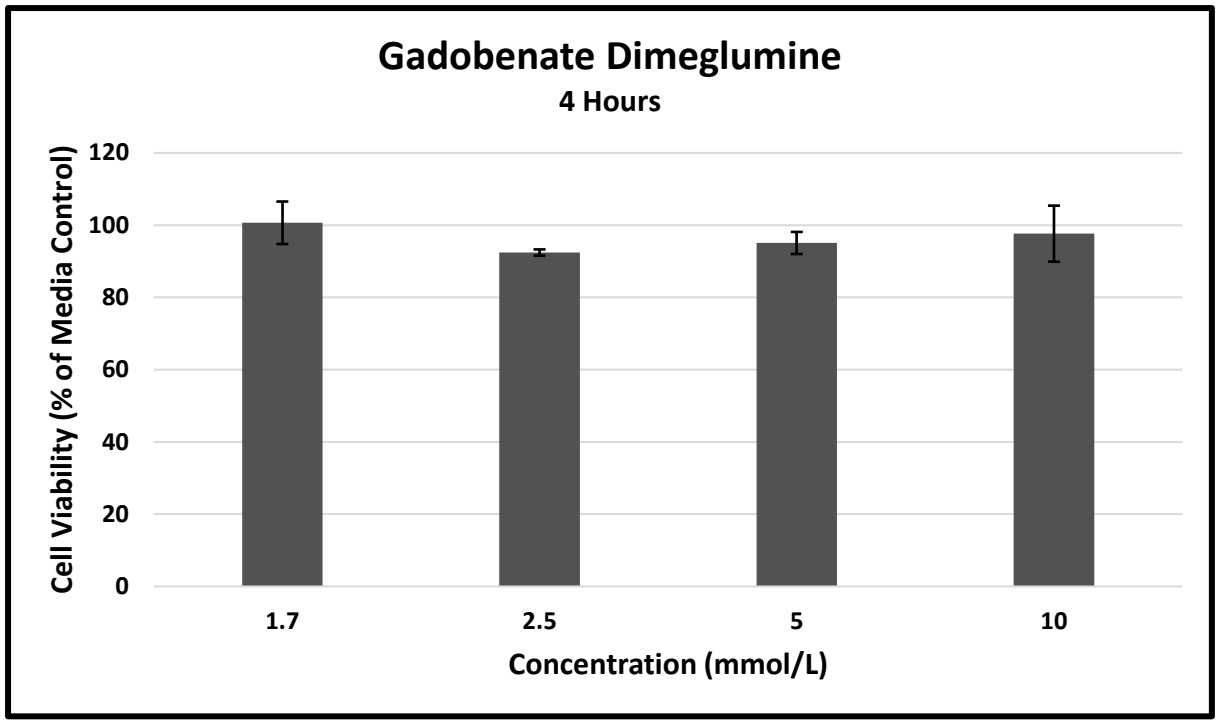

Figure 3.27 Bar graph illustrating the effect of treatment with varying concentrations of Gadobenate Dimeglumine for 4 hours on cell viability of primary human chondrocytes using the MTS assay. Data represents six independent experiments.

3.6.1.3 Gadobenate Dimeglumine: 16 Hours

A Shapiro-Wilks test and Kolmogorov-Smirnov test ($p > 0.05$) and a visual inspection of their histograms, normal Q-Q plots and box plots showed that cell viability scores were approximately normally distributed for each concentration, with a skewness of 1.36 (SE = 1.01) and a kurtosis of 2.56 (SE = 2.62) for 1.7 mmol/L , a skewness of 0.46 (SE = 0.91) and a kurtosis of -1.25 (SE = 2.0) for 2.5 mmol/L, a skewness of 0.46 (SE = 0.91) and a kurtosis of -1.71 (SE = 2.0) for 5 mmol/L and a skewness of 0.04 (SE = 0.91) and a kurtosis of -2.53 (SE = 2.0) for 10 mmol/L.

At 16 hours there was no significant difference in cell viability between the media control and a Gadobenate Dimeglumine concentration of either 1.7 mmol/L (p = 1.0; 95% CI, -2.6 – 7.8), 2.5 mmol/L (p = 0.07; 95% CI, -0.1 – 9.9), 5 mmol/L (p = 1.0; 95% CI, -0.6 – 9.2) or 10 mmol/L (p = 1.0; 95% CI, -3.3 – 6.5).

There was no significant concentration-dependent effect of Gadobenate Dimeglumine on cell viability ([1.7 – 2.5 mmol/L, p = 1.0; 95% CI, -2.8 – 7.6], [1.7 – 5 mmol/L, p = 1.0; 95% CI, -3.5 – 6.9], [1.7 – 10 mmol/L, p = 1.0; 95% CI, -4.2 – 6.2], [2.5 – 5 mmol/L, p = 1.0; 95% CI, -4.2 – 5.6], [2.5 – 10 mmol/L, p = 1.0; 95% CI, -1.5 – 8.3], [5 – 10 mmol/L, p = 1.0; 95% CI, -2.2 – 7.6]).

Table 3.28 Effect of treatment with varying concentrations of Gadobenate Dimeglumine for 16 hours on cell viability of primary human chondrocytes using the MTS assay. Data represents six independent experiments.

	1.7 mmol/L	**2.5 mmol/L**	**5 mmol/L**	**10 mmol/L**	***p*-value**
Mean	97.4	95.0	95.7	98.4	
95% CI	91.6 – 103.2	92.2 – 97.9	92.5 – 98.9	95.3 – 101.5	
SD	3.6	2.3	2.7	2.5	
Range	94.0 – 102.6	92.4 – 98.1	92.8 – 99.2	95.6 – 101.3	
					p = 0.29

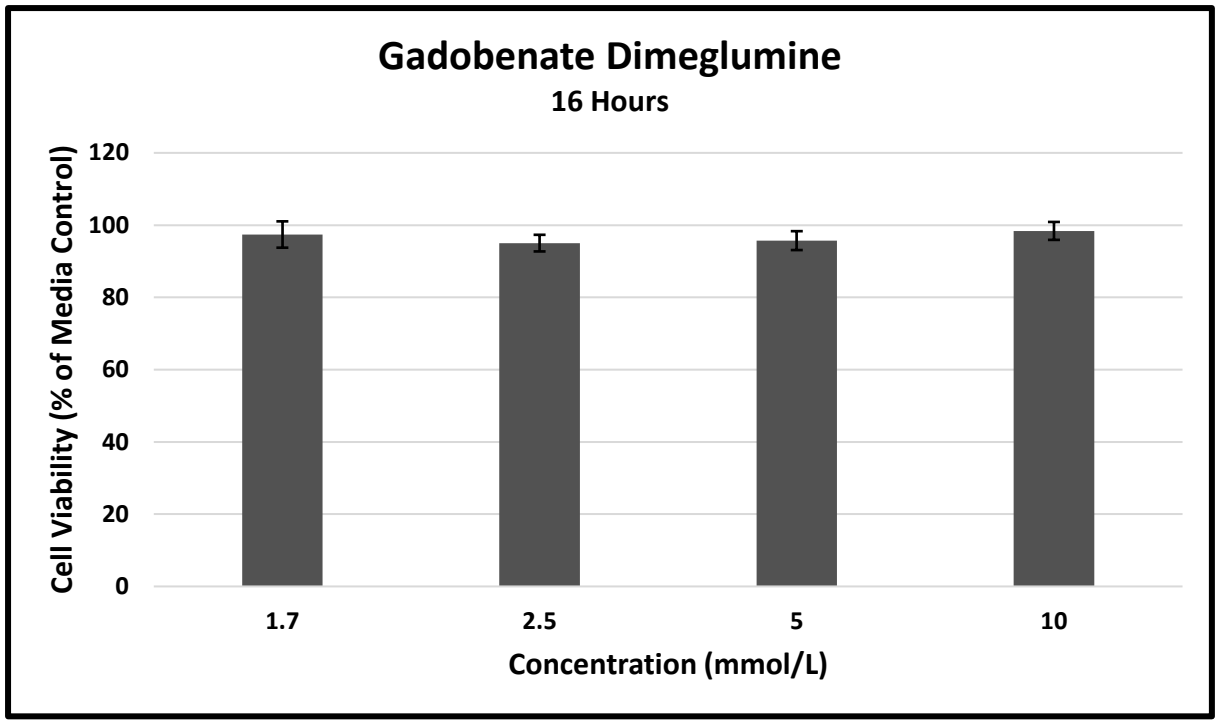

Figure 3.28 Bar graph illustrating the effect of treatment with varying concentrations of Gadobenate Dimeglumine for 16 hours on cell viability of primary human chondrocytes using the MTS assay. Data represents six independent experiments.

3.6.1.4 Gadobenate Dimeglumine: 24 Hours

A Shapiro-Wilks test and Kolmogorov-Smirnov test ($p > 0.05$) and a visual inspection of their histograms, normal Q-Q plots and box plots showed that cell viability scores were approximately normally distributed for each concentration, with a skewness of 1.0 (SE = 1.0) and a kurtosis of 2.0 (SE = 2.6) for 1.7 mmol/L , a skewness of -1.1 (SE = 0.91) and a kurtosis of 2.0 (SE = 2.0) for 2.5 mmol/L, a skewness of 0.6 (SE = 0.91) and a kurtosis of 0.45 (SE = 2.0) for 5 mmol/L and a skewness of 1.9 (SE = 0.85) and a kurtosis of 4.1 (SE = 1.7) for 10 mmol/L.

At 24 hours there was no significant difference in cell viability between the media control and a Gadobenate Dimeglumine concentration of either 1.7 mmol/L (p = 1.0; 95% CI, -9.0 – 12.9), 2.5 mmol/L (p = 1.0; 95% CI, -8.1 – 12.5), 5 mmol/L (p = 1.0; 95% CI, -8.2 – 12.4) or 10 mmol/L (p = 1.0; 95% CI, -6.8 – 12.8).

There was no significant concentration-dependent effect of Gadobenate Dimeglumine on cell viability ([1.7 – 2.5 mmol/L, p = 1.0; 95% CI, -7.3 – 15.5], [1.7 – 5 mmol/L, p = 1.0; 95% CI, -7.4 – 15.4], [1.7 – 10 mmol/L, p = 1.0; 95% CI, -6.1 – 15.9], [2.5 – 5 mmol/L, p = 1.0; 95% CI, -10.6 – 10.9], [2.5 – 10 mmol/L, p = 1.0; 95% CI, -9.5 – 11.1], [5 – 10 mmol/L, p = 1.0; 95% CI, -9.4 – 11.2]).

Table 3.29 Effect of treatment with varying concentrations of Gadobenate Dimeglumine for 24 hours on cell viability of primary human chondrocytes using the MTS assay. Data represents six independent experiments

	1.7 mmol/L	**2.5 mmol/L**	**5 mmol/L**	**10 mmol/L**	***p*-value**
Mean	101.9	97.8	97.0	97.0	
95% CI	98.1 – 105.8	86.5 – 109.1	90.3 – 103.7	90.3 – 103.7	
SD	2.4	9.1	6.4	6.4	
Range	99.4 – 105.3	83.3 – 107.9	91.2 – 109.3	91.2 – 109.3	
					p = 0.64

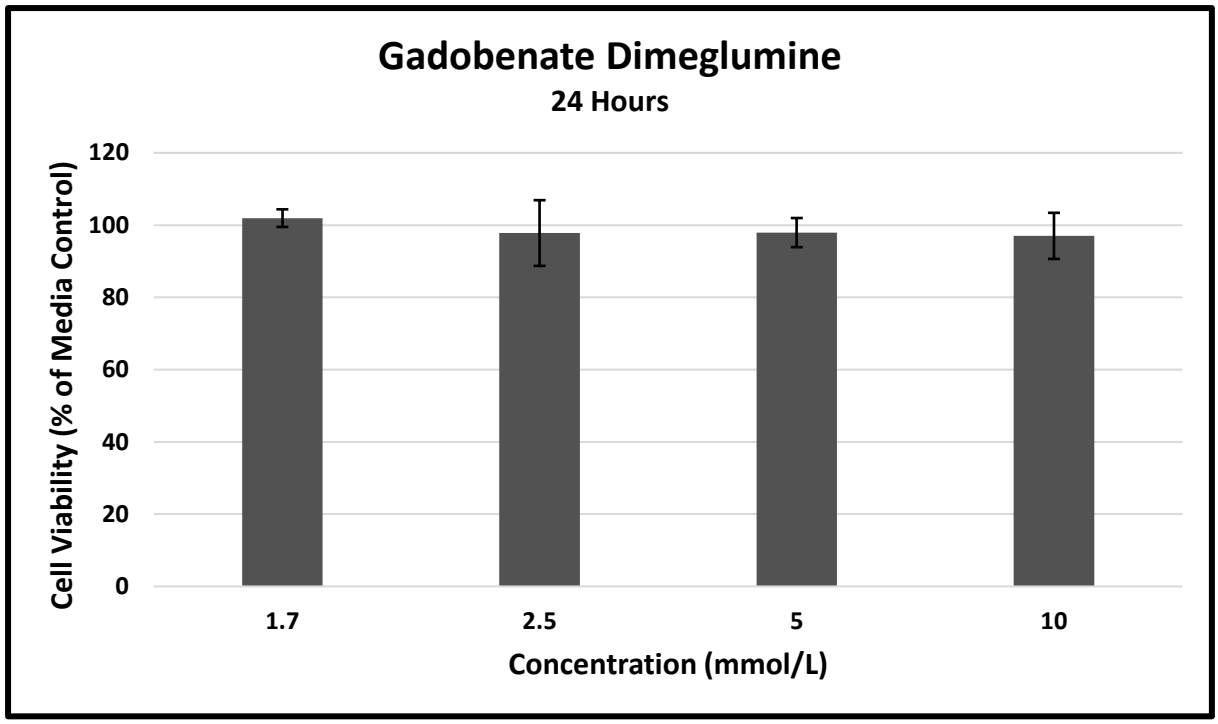

Figure 3.29 Bar graph illustrating the effect of treatment with varying concentrations of Gadobenate Dimeglumine for 24 hours on cell viability of primary human chondrocytes using the MTS assay. Data represents six independent experiments.

3.6.2 Concentration-dependent effect of Gadobenate Dimeglumine on primary human chondrocytes

3.6.2.1 Gadobenate Dimeglumine: 1.7 mmol/L (1:300)

A Shapiro-Wilks test and Kolmogorov-Smirnov test ($p > 0.05$) and a visual inspection of their histograms, normal Q-Q plots and box plots showed that cell viability scores were approximately normally distributed for each time point, with a skewness of -1.24 (SE = 1.01) and a kurtosis of 1.75 (SE = 2.62) for 1 hour, a skewness of 1.72 (SE = 1.23) and a kurtosis of 1.75 (SE = 2.65) for 4 hours, a skewness of 1.36 (SE = 1.01) and a kurtosis of 2.56 (SE = 2.62) for 16 hours and a skewness of 1.0 (SE = 1.01) and a kurtosis of 2.04 (SE = 2.62) for 24 hours.

There was no significant time-dependent effect of Gadobenate Dimeglumine1.7 mmol/L on cell viability ([1 hour – 4 hours, $p = 1.0$; 95% CI, -5.1 – 12.3], [1 hour – 16 hours, $p = 1.0$; 95% CI, -7.7 – 8.4], [1 hour – 24 hours, $p = 0.46$; 95% CI, -3.1 – 12.9]), [4 hours – 16 hours, $p = 1.0$; 95% CI, -5.4 – 11.9]), [4 hours – 24 hours, $p = 1.0$; 95% CI, -7.4 – 9.9]) and [16 hours – 24 hours, $p = 0.58$; 95% CI, -3.5 – 12.6]).

Table 3.30 Effect of treatment with Gadobenate Dimeglumine (1.7 mmol/L; 1:300 *v:v*) for varying time periods on cell viability of primary human chondrocytes using the MTS assay. Data represents six independent experiments.

	1 Hour	**4 Hours**	**16 Hours**	**24 Hours**	***p*-value**
Mean	97.1	100.7	97.4	101.9	
95% CI	94.0 – 100.1	86.1 – 115.3	91.6 – 103.2	98.1 – 105.8	
SD	1.9	5.9	3.6	2.4	
Range	94.4 – 98.8	97.1 – 107.4	94.0 – 102.6	99.4 – 105.3	
					$p = 0.21$

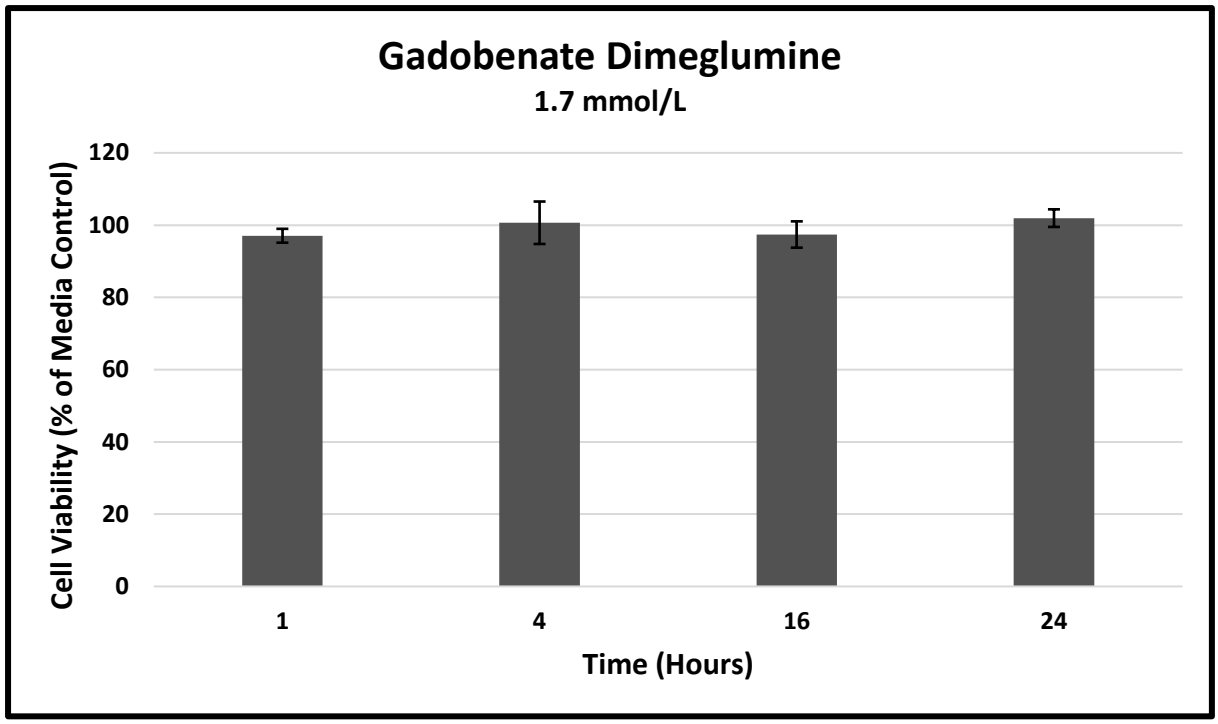

Figure 3.30 Bar graph illustrating the effect of treatment with Gadobenate Dimeglumine (1.7 mmol/L; 1:300 *v:v*) for varying time periods on cell viability of primary human chondrocytes using the MTS assay. Data represents six independent experiments.

3.6.2.2 Gadobenate Dimeglumine: 2.5 mmol/L (1:200)

A Shapiro-Wilks test and Kolmogorov-Smirnov test ($p > 0.05$) and a visual inspection of their histograms, normal Q-Q plots and box plots showed that cell viability scores were approximately normally distributed for each time point, with a skewness of -0.98 (SE = 0.91) and a kurtosis of 0.87 (SE = 2.0) for 1 hour, a skewness of 1.22 (SE = 1.01) and a kurtosis of 2.23 (SE = 2.62) for 4 hours, a skewness of 0.46 (SE = 0.92) and a kurtosis of -1.25 (SE = 2.0) for 16 hours and a skewness of -1.09 (SE = 0.91) and a kurtosis of 2.03 (SE = 2.0) for 24 hours.

There was no significant time-dependent effect of Gadobenate Dimeglumine 2.5 mmol/L on cell viability ([1 hour – 4 hours, $p = 0.44$; 95% CI, -4.0 – 17.9], [1 hour – 16 hours, $p = 1.0$; 95% CI, -6.0 – 14.7], [1 hour – 24 hours, $p = 1.0$; 95% CI, -8.8 – 11.9]), [4 hours – 16 hours, $p = 1.0$; 95% CI, -8.4 – 13.6]), [4 hours – 24 hours, $p = 1.0$; 95% CI, -5.6 – 16.4]) and [16 hours – 24 hours, $p = 1.0$; 95% CI, -7.6 – 13.2]).

Table 3.31 Effect of treatment with Gadobenate Dimeglumine (2.5 mmol/L; 1:200 *v:v*) for varying time periods on cell viability of primary human chondrocytes using the MTS assay. Data represents six independent experiments.

	1 Hour	**4 Hours**	**16 Hours**	**24 Hours**	***p*-value**
Mean	99.4	92.4	95.0	97.8	
95% CI	93.7 – 105.1	91.1 – 93.8	92.2 – 97.9	86.5 – 109.1	
SD	4.6	0.9	2.3	9.1	
Range	92.3 – 104.3	91.6 – 96.6	92.4 – 98.1	83.3 – 107.9	
					$p = 0.27$

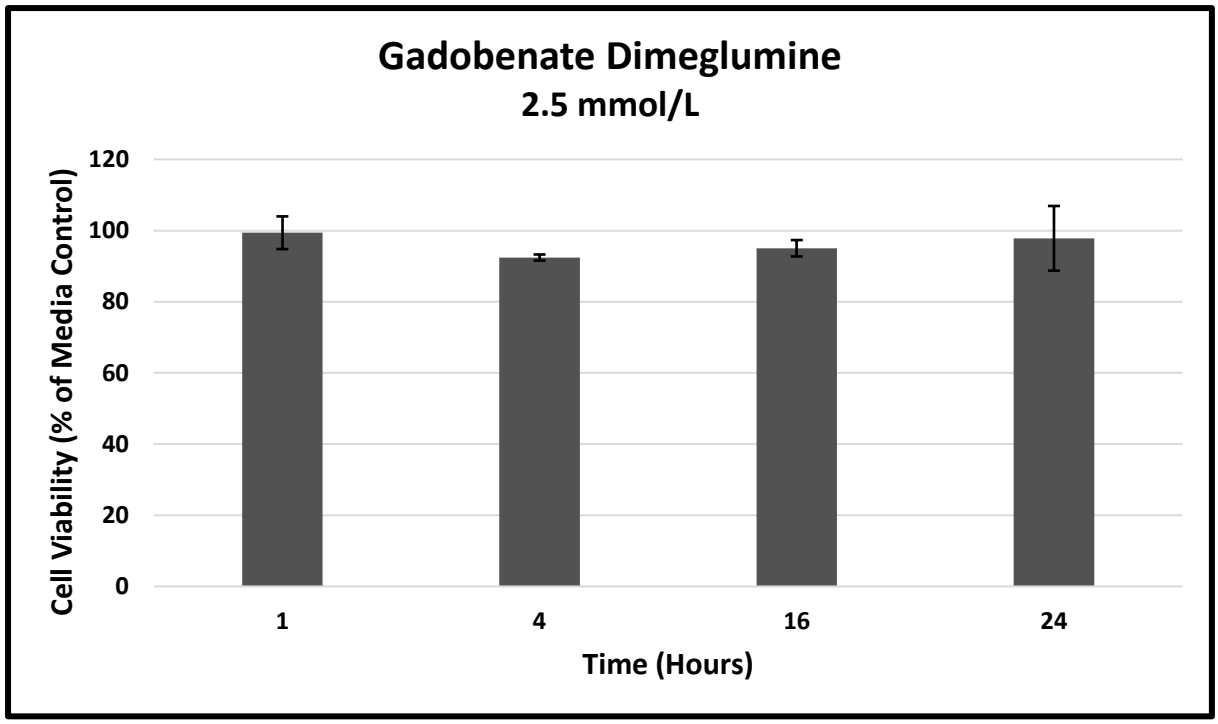

Figure 3.31 Bar graph illustrating the effect of treatment with Gadobenate Dimeglumine (2.5 mmol/L; 1:200 *v:v*) for varying time periods on cell viability of primary human chondrocytes using the MTS assay. Data represents six independent experiments.

3.6.2.3 Gadobenate Dimeglumine: 5 mmol/L (1:100)

A Shapiro-Wilks test and Kolmogorov-Smirnov test ($p > 0.05$) and a visual inspection of their histograms, normal Q-Q plots and box plots showed that cell viability scores were approximately normally distributed for each time point, with a skewness of -0.21 (SE = 0.91) and a kurtosis of -0.82 (SE = 2.0) for 1 hour, a skewness of 0.08 (SE = 1.01) and a kurtosis of -5.04 (SE = 2.62) for 4 hours, a skewness of 0.46 (SE = 0.91) and a kurtosis of 1.71 (SE = 2.0) for 16 hours and a skewness of 0.59 (SE = 0.91) and a kurtosis of 0.45 (SE = 2.0) for 24 hours.

There was no significant time-dependent effect of Gadobenate Dimeglumine5 mmol/L on cell viability ([1 hour – 4 hours, $p = 0.99$; 95% CI, -4.3 – 12.2], [1 hour – 16 hours, $p = 1.0$; 95% CI, -4.5 – 11.1], [1 hour – 24 hours, $p = 1.0$; 95% CI, -6.7 – 8.8]), [4 hours – 16 hours, $p = 1.0$; 95% CI, -7.6 – 8.9]), [4 hours – 24 hours, $p = 1.0$; 95% CI, -5.4 – 11.1]) and [16 hours – 24 hours, $p = 1.0$; 95% CI, -5.5 – 9.9]).

Table 3.32 Effect of treatment with Gadobenate Dimeglumine (5 mmol/L; 1:100 *v:v*) for varying time periods on cell viability of primary human chondrocytes using the MTS assay. Data represents six independent experiments.

	1 Hour	**4 Hours**	**16 Hours**	**24 Hours**	***p*-value**
Mean	99.0	95.1	95.7	97.0	
95% CI	92.1 – 105.9	90.2 – 99.9	92.5 – 98.9	90.3 – 103.7	
SD	5.6	3.1	2.7	6.4	
Range	91.5 – 105.8	92.1 – 98.3	92.8 – 99.2	91.2 – 109.3	
					$p = 0.43$

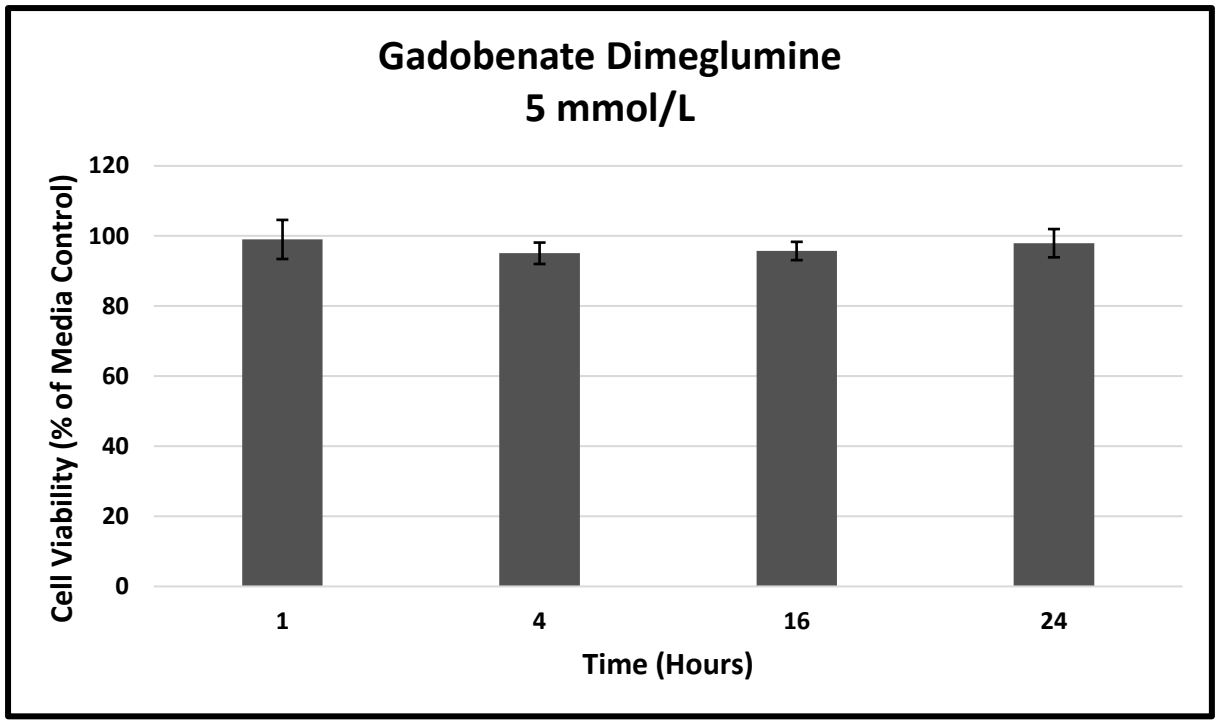

Figure 3.32 Bar graph illustrating the effect of treatment with Gadobenate Dimeglumine (5 mmol/L; 1:100 *v:v*) for varying time periods on cell viability of primary human chondrocytes using the MTS assay. Data represents six independent experiments.

3.6.2.4 Gadobenate Dimeglumine: 10 mmol/L (1:50)

A Shapiro-Wilks test and Kolmogorov-Smirnov test ($p > 0.05$) and a visual inspection of their histograms, normal Q-Q plots and box plots showed that cell viability scores were approximately normally distributed for each time point, with a skewness of -0.54 (SE = 0.91) and a kurtosis of -1.1 (SE = 2.0) for 1 hour, a skewness of 1.84 (SE = 1.01) and a kurtosis of 3.5 (SE = 2.62) for 4 hours, a skewness of 0.04 (SE = 0.91) and a kurtosis of -2.53 (SE = 2.0) for 16 hours and a skewness of 1.86 (SE = 0.85) and a kurtosis of 4.05 (SE = 1.74) for 24 hours.

There was no significant time-dependent effect of Gadobenate Dimeglumine10 mmol/L on cell viability ([1 hour – 4 hours, $p = 1.0$; 95% CI, -10.0 – 12.4], [1 hour – 16 hours, $p = 1.0$; 95% CI, -8.6 – 12.5], [1 hour – 24 hours, $p = 1.0$; 95% CI, -9.6 – 10.7]), [4 hours – 16 hours, $p = 1.0$; 95% CI, -10.5 – 12.0]), [4 hours – 24 hours, $p = 1.0$; 95% CI, -10.2 – 11.4]) and [16 hours – 24 hours, $p = 1.0$; 95% CI, -8.8 – 11.5]).

Table 3.33 Effect of treatment with Gadobenate Dimeglumine (10 mmol/L; 1:50 *v:v*) for varying time periods on cell viability of primary human chondrocytes using the MTS assay. Data represents six independent experiments.

	1 Hour	**4 Hours**	**16 Hours**	**24 Hours**	***p*-value**
Mean	96.4	97.6	98.4	97.0	
95% CI	90.7 – 102.2	85.3 – 109.9	95.3 – 101.5	90.3 – 103.7	
SD	4.7	7.8	2.5	6.4	
Range	89.9 – 101.0	92.2 – 109.1	95.6 – 101.3	91.2 – 109.3	
					$p = 0.95$

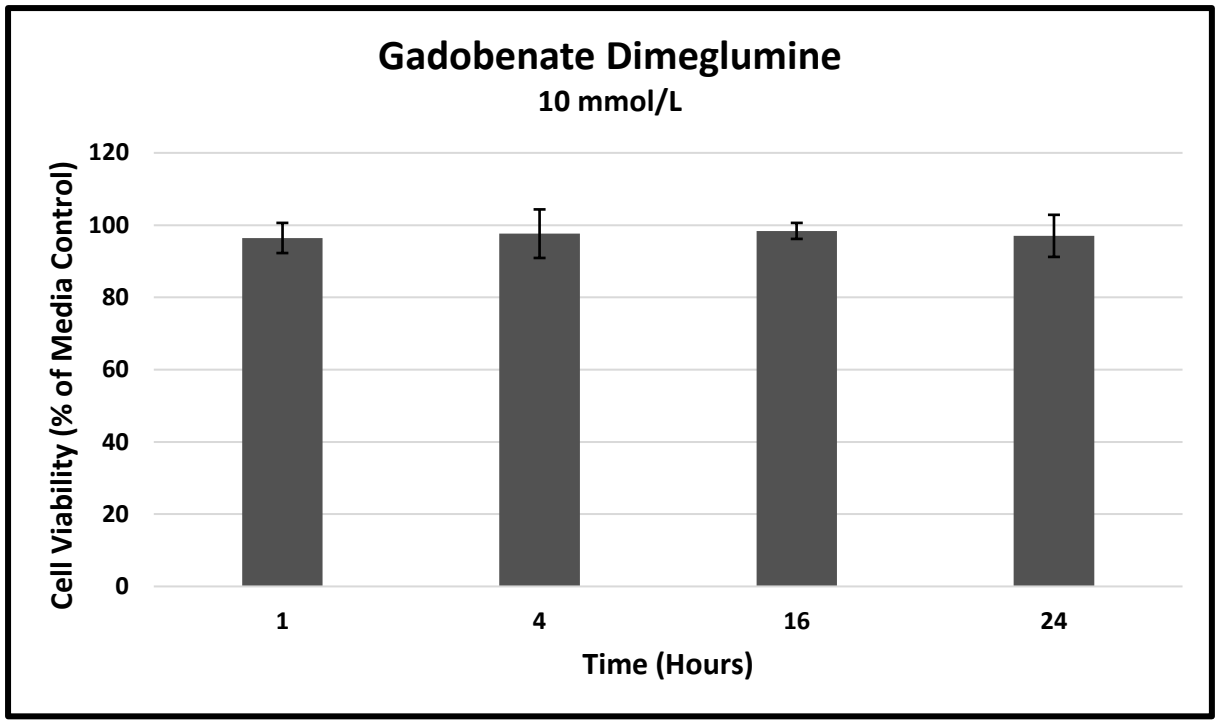

Figure 3.33 Bar graph illustrating the effect of treatment with Gadobenate Dimeglumine (10 mmol/L; 1:50 *v:v*) for varying time periods on cell viability of primary human chondrocytes using the MTS assay. Data represents six independent experiments.

3.7 The effect of an MR Arthrogram Clinical Solution on primary human chondrocytes

To investigate the effect of individual agents, and combinations of agents, of an MR Arthrogram Clinical Solution on primary human chondrocyte cell viability, cells were treated with varying agents, either alone or in combination with other agents [either Gadopentetate Dimeglumine (2.5 mmol/L), Lidocaine (0.5%), Gadopentetate Dimeglumine (2.5 mmol/L) + Lidocaine (0.5%) or Lidocaine (1%)] for 30 minutes, 45 minutes and 60 minutes (Section 2.3.5.3). Shorter time periods were selected as solutions were made without cell media. Cells exposed to normal saline served as the controls. Cell viability was then assessed using the MTS assay (Section 2.3.6.1) and crystal violet staining (Section 2.3.6.2). All data represents the results of six independent experiments.

3.7.1 MR Arthrogram Clinical Solution: The effect of individual agents, and combinations of agents, on primary human chondrocyte viability using an MTS assay

3.7.1.1 MR Arthrogram Clinical Solution: 30 Minutes

A Shapiro-Wilks test and Kolmogorov-Smirnov test ($p > 0.05$) and a visual inspection of their histograms, normal Q-Q plots and box plots showed that cell viability scores were approximately normally distributed for each solution, with a skewness of 0.29 (SE = 0.47) and a kurtosis of 0.3 (SE = 0.92) for Gadopentetate Dimeglumine (2.5 mmol/L), a skewness of 0.13 (SE = 0.46) and a kurtosis of -0.38 (SE = 0.90) for Lidocaine (0.5%), a skewness of 0.64 (SE = 0.46) and a kurtosis of -0.41 (SE = 0.89) for Gadopentetate Dimeglumine (2.5 mmol/L) + Lidocaine (0.5%) and a skewness of 0.28 (SE = 0.47) and a kurtosis of -0.36 (SE = 0.92) for Lidocaine (1%).

At 30 minutes there was no significant difference in cell viability between the saline control and Gadopentetate Dimeglumine (2.5 mmol/L) ($p = 1.0$; 95% CI, -13.8 – 23.2). There was a significant difference in cell viability between the saline control and Lidocaine (0.5%) ($p < 0.001$; 95% CI, 11.1 – 47.8), Gadopentetate Dimeglumine (2.5 mmol/L) + Lidocaine (0.5%) ($p <0.001$; 95% CI, 15.3 – 51.6) and Lidocaine 1% ($p < 0.001$; 95% CI, 26.1 – 63.2).

A significant inter-solution difference was found between Gadopentetate Dimeglumine (2.5 mmol/L) and each of Lidocaine 0.5% ($p = 0.002$; 95% CI, 6.2 – 43.3), Gadopentetate Dimeglumine (2.5 mmol/L) + Lidocaine (0.5%) ($p <0.001$; 95% CI, 10.4 – 47.1) and Lidocaine 1% ($p < 0.001$; 95% CI, 21.3 – 58.7).

The remaining inter-solution comparisons did not reach significance:

- Lidocaine 0.5% - Gadopentetate Dimeglumine (2.5 mmol/L) + Lidocaine (0.5%) (p = 1.0; 95% CI, -14.1 – 22.2).
- Lidocaine 0.5 % - Lidocaine 1% (p = 0.2; 95% CI, -3.3 – 33.8).
- Gadopentetate Dimeglumine (2.5 mmol/L) + Lidocaine (0.5%) – Lidocaine 1% (p = 0.83; 95% CI, -7.12 – 29.6).

Table 3.34 Effect of treatment of individual agents, and combinations of agents, of an MR Arthrogram Clinical Solution for 30 minutes on cell viability of primary human chondrocytes using the MTS assay. Data represents six independent experiments

	Gadopentetate Dimeglumine (2.5 mmol/L)	**Lidocaine (0.5%)**	**Gadopentetate Dimeglumine (2.5 mmol/L) + Lidocaine (0.5%)**	**Lidocaine (1%)**	***p*-value**
Mean	95.2	72.5	72.9	63.8	
95% CI	83.6 – 106.9	60.0 – 85.6	54.7 – 78.9	47.7 - 72	
SD	5.6	5.3	7.8	9.75	
Range	82.1 – 113.8	61.4 – 87.1	62.8 – 93.4	44.3 – 83.4	
					$p < 0.001$

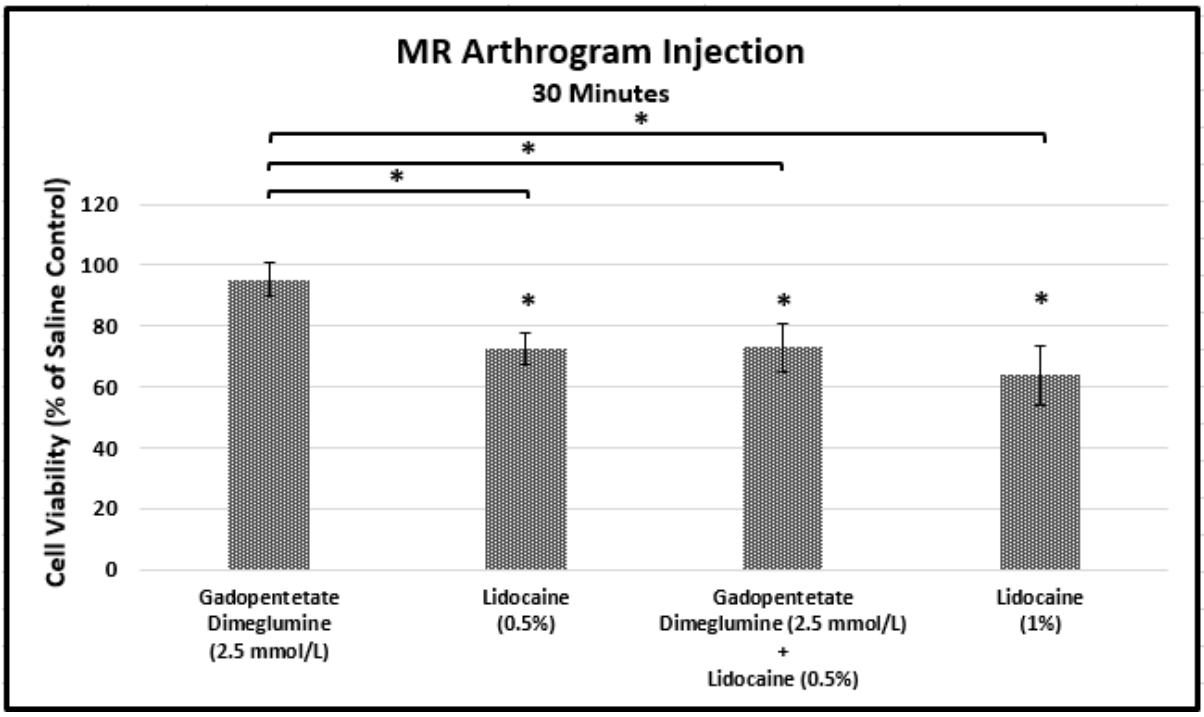

Figure 3.34 Bar graph illustrating the effect of treatment of individual agents, and combinations of agents, of an MR Arthrogram Clinical Solution for 30 minutes on cell viability of primary human chondrocytes using the MTS assay. Data represents six independent experiments

* Statistically significant difference in cell viability from saline control

* Statistically significant inter-solution difference in cell viability

3.7.1.2 MR Arthrogram Clinical Solution: 45 Minutes

A Shapiro-Wilks test and Kolmogorov-Smirnov test ($p > 0.05$) and a visual inspection of their histograms, normal Q-Q plots and box plots showed that cell viability scores were approximately normally distributed for each solution, with a skewness of -0.13 (SE = 0.52) and a kurtosis of -0.71 (SE = 1.01) for Gadopentetate Dimeglumine (2.5 mmol/L), a skewness of 0.20 (SE = 0.49) and a kurtosis of -0.04 (SE = 0.95) for Lidocaine (0.5%), a skewness of 0.22 (SE = 0.43) and a kurtosis of 0.47 (SE = 0.83) for Gadopentetate Dimeglumine (2.5 mmol/L) + Lidocaine (0.5%) and a skewness of 0.71 (SE = 0.43) and a kurtosis of -0.41 (SE = 0.83) for Lidocaine (1%).

At 45 minutes there was no significant difference in cell viability between the saline control and Gadopentetate Dimeglumine (2.5 mmol/L) ($p = 1.0$; 95% CI, -16.5 – 18.6). There was a significant difference in cell viability between the saline control and Lidocaine (0.5%) ($p < 0.001$; 95% CI, 14.4 – 58.1), Gadopentetate Dimeglumine (2.5 mmol/L) + Lidocaine (0.5%) ($p <0.001$; 95% CI, 25 – 56.2) and Lidocaine 1% ($p < 0.001$; 95% CI, 36.0 – 67.3).

A significant inter-solution difference was found between Gadopentetate Dimeglumine (2.5 mmol/L) and each of Lidocaine 0.5% ($p < 0.001$; 95% CI, 22.2 – 58.3), Gadopentetate Dimeglumine (2.5 mmol/L) + Lidocaine (0.5%) ($p <0.001$; 95% CI, 22.7 – 56.5) and Lidocaine 1% ($p < 0.001$; 95% CI, 33.7 – 67.5).

The remaining inter-solution comparisons did not reach significance:

- Lidocaine 0.5% - Gadopentetate Dimeglumine (2.5 mmol/L) + Lidocaine (0.5%) ($p = 1.0$; 95% CI, -15.5 – 16.8).
- Lidocaine 0.5 % - Lidocaine 1% ($p = 0.69$; 95% CI, -5.8 – 26.6).
- Gadopentetate Dimeglumine (2.5 mmol/L) + Lidocaine (0.5%) – Lidocaine 1% ($p = 0.36$; 95% CI, -3.8 – 25.9).

Table 3.35 Effect of treatment of individual agents, and combinations of agents, of an MR Arthrogram Clinical Solution for 45 minutes on cell viability of primary human chondrocytes using the MTS assay. Data represents six independent experiments

	Gadopentetate Dimeglumine (2.5 mmol/L)	**Lidocaine (0.5%)**	**Gadopentetate Dimeglumine (2.5 mmol/L) + Lidocaine (0.5%)**	**Lidocaine (1%)**	***p*-value**
Mean	102.7	60.0	57.5	45.9	
95% CI	88.3 – 117.0	38.9 – 75.0	40.3 – 75.0	30.5 – 61.3	
SD	11.5	11.4	14.0	12.4	
Range	87.3 – 118.2	47.4 – 70.6	44.3 – 79.5	29.0 – 63.8	
					$p < 0.001$

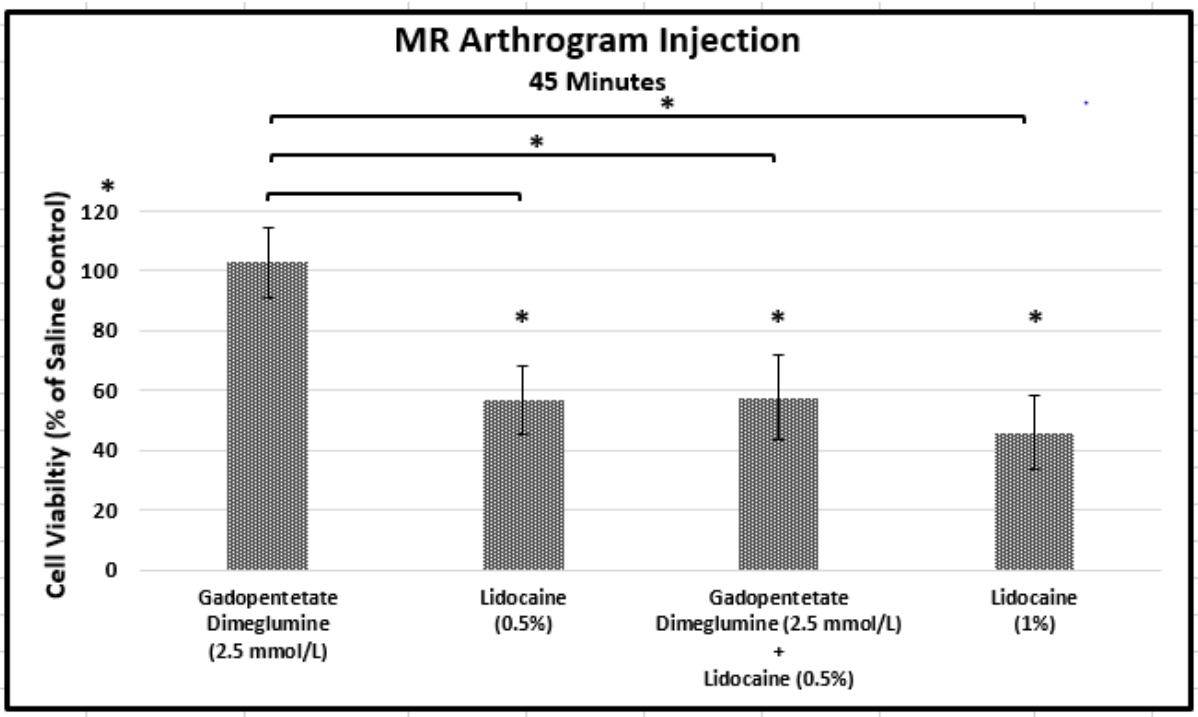

Figure 3.35 Bar graph illustrating the effect of treatment of individual agents, and combinations of agents, of an MR Arthrogram Clinical Solution for 45 minutes on cell viability of primary human chondrocytes using the MTS assay. Data represents six independent experiments

* Statistically significant difference in cell viability from saline control

* Statistically significant inter-solution difference in cell viability

3.7.1.3 MR Arthrogram Clinical Solution: 60 Minutes

A Shapiro-Wilks test and Kolmogorov-Smirnov test ($p > 0.05$) and a visual inspection of their histograms, normal Q-Q plots and box plots showed that cell viability scores were not normally distributed for two solutions, namely Gadopentetate Dimeglumine (2.5 mmol/L) + Lidocaine (0.5%) and Lidocaine 1%. There was a skewness of 0.98 (SE = 0.46) and a kurtosis of 1.11 (SE = 0.89) for Gadopentetate Dimeglumine (2.5 mmol/L), a skewness of 0.76 (SE = 0.44) and a kurtosis of 0.47 (SE = 0.86) for Lidocaine (0.5%), a skewness of 0.94 (SE = 0.43) and a kurtosis of 0.24 (SE = 0.85) for Gadopentetate Dimeglumine (2.5 mmol/L) + Lidocaine (0.5%) and a skewness of 0.96 (SE = 0.39) and a kurtosis of 0.13 (SE = 0.77) for Lidocaine (1%).

At 60 minutes there was no significant difference in cell viability between the saline control and Gadopentetate Dimeglumine (2.5 mmol/L) ($p = 1.0$; SE 11.6). There was a significant difference in cell viability between the saline control and Lidocaine (0.5%) ($p < 0.001$; SE 11.4), Gadopentetate Dimeglumine (2.5 mmol/L) + Lidocaine (0.5%) ($p <0.001$; SE 11.3) and Lidocaine 1% ($p < 0.001$; SE 10.7).

A significant inter-clinical solution difference was found between Gadopentetate Dimeglumine (2.5 mmol/L) and each of Lidocaine 0.5% ($p = 0.002$; SE 11.7), Gadopentetate Dimeglumine (2.5 mmol/L) + Lidocaine (0.5%) ($p <0.001$; SE 11.6) and Lidocaine 1% ($p < 0.001$; SE 11.0).

The remaining inter-solution comparisons did not reach significance:

- Lidocaine 0.5% - Gadopentetate Dimeglumine (2.5 mmol/L) + Lidocaine (0.5%) ($p = 1.0$; SE 11.4).
- Lidocaine 0.5 % - Lidocaine 1% ($p = 0.2$; SE 10.8).
- Gadopentetate Dimeglumine (2.5 mmol/L) + Lidocaine (0.5%) – Lidocaine 1% ($p = 0.48$; SE 10.7).

Table 3.36 Effect of treatment of individual agents, and combinations of agents, of an MR Arthrogram Clinical Solution for 60 minutes on cell viability of primary human chondrocytes using the MTS assay. Data represents six independent experiments

	Gadopentetate Dimeglumine (2.5 mmol/L)	**Lidocaine (0.5%)**	**Gadopentetate Dimeglumine (2.5 mmol/L) + Lidocaine (0.5%)**	**Lidocaine (1%)**	***p*-value**
Mean	87.1	55.6	54.3	42.0	
95% CI	79.1 – 95.1	42.9 – 68.3	36.1 – 72.4	17.8 – 66.2	
SD	7.6	12.1	14.6	15.2	
Range	72.2 – 91.5	38.5 – 75.1	41.0 – 76.1	20.5 – 55.7	
					$p < 0.001$

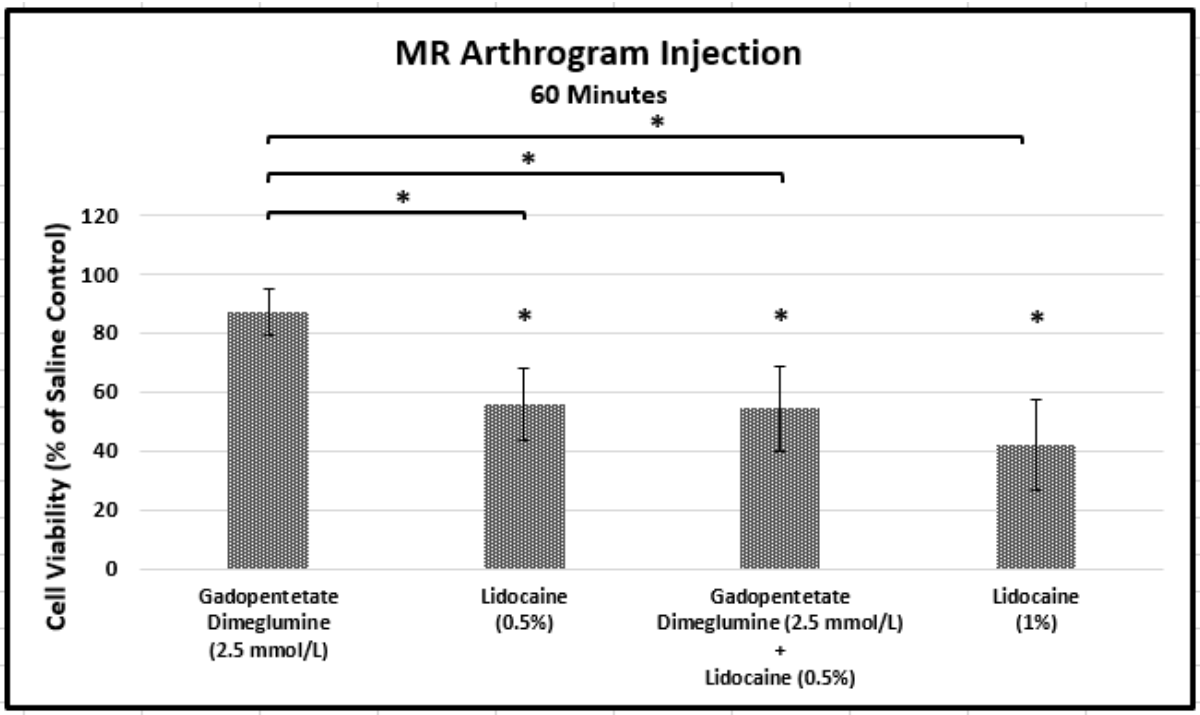

Figure 3.36 Bar graph illustrating the effect of treatment of individual agents, and combinations of agents, of an MR Arthrogram Clinical Solution for 60 minutes on cell viability of primary human chondrocytes using the MTS assay. Data represents six independent experiments

* Statistically significant difference in cell viability from saline control

* Statistically significant inter-solution difference in cell viability

3.7.2 MR Arthrogram Clinical Solution: The effect of individual agents, and combinations of agents, on primary human chondrocyte viability using crystal violet staining

3.7.2.1 MR Arthrogram Clinical Solution: 30 Minutes

A Shapiro-Wilks test and Kolmogorov-Smirnov test ($p > 0.05$) and a visual inspection of their histograms, normal Q-Q plots and box plots showed that cell viability scores were not normally distributed for each solution, with a skewness of 0.28 (SE = 0.54) and a kurtosis of -0.59 (SE = 1.04) for Gadopentetate Dimeglumine (2.5 mmol/L), a skewness of -0.36 (SE = 0.62) and a kurtosis of 0.63 (SE = 1.19) for Lidocaine (0.5%), a skewness of 0.95 (SE = 0.59) and a kurtosis of 0.39 (SE = 1.15) for Gadopentetate Dimeglumine (2.5 mmol/L) + Lidocaine (0.5%) and a skewness of -0.29 (SE = 0.62) and a kurtosis of 0.87 (SE = 1.19) for Lidocaine (1%).

At 30 minutes there was no significant difference in cell viability between the saline control and Gadopentetate Dimeglumine (2.5 mmol/L) ($p = 1.0$; 95% SE 7.3). There was a significant difference in cell viability between the saline control and Lidocaine (0.5%) ($p < 0.001$; SE 7.9), {Gadopentetate Dimeglumine (2.5 mmol/L) + Lidocaine (0.5%)} ($p <0.001$; SE 7.8) and Lidocaine (1%) ($p < 0.001$; SE 8.0).

A significant inter-solution difference was found between Gadopentetate Dimeglumine (2.5 mmol/L) and each of Lidocaine 0.5% ($p = 0.004$; SE 7.9), Gadopentetate Dimeglumine (2.5 mmol/L) + Lidocaine (0.5%) ($p = 0.005$; SE 7.8) and Lidocaine 1% ($p < 0.001$; SE 7.6).

The remaining inter-solution comparisons did not reach significance:

- Lidocaine 0.5% - Gadopentetate Dimeglumine (2.5 mmol/L) + Lidocaine (0.5%) ($p = 1.0$; SE 8.4).
- Lidocaine 0.5 % - Lidocaine 1% ($p = 0.2$; SE 8.6).
- Gadopentetate Dimeglumine (2.5 mmol/L) + Lidocaine (0.5%) – Lidocaine 1% ($p = 0.83$; SE 8.4).

Table 3.37 Effect of treatment of individual agents, and combinations of agents, of an MR Arthrogram Clinical Solution for 30 minutes on cell viability of primary human chondrocytes using crystal violet staining. Data represents six independent experiments

	Gadopentetate Dimeglumine (2.5 mmol/L)	**Lidocaine (0.5%)**	**Gadopentetate Dimeglumine (2.5 mmol/L) + Lidocaine (0.5%)**	**Lidocaine (1%)**	***p*-value**
Mean	97.4	70.0	71.5	64.9	
95% CI	92.4 – 102.4	63.1 – 77.0	62.5 – 81.4	58.0 – 71.8	
SD	9.9	11.5	16.4	11.4	
Range	87.3 – 105.1	61.4 – 79.2	62.0 – 95.0	49.0 – 89.5	
					$p < 0.001$

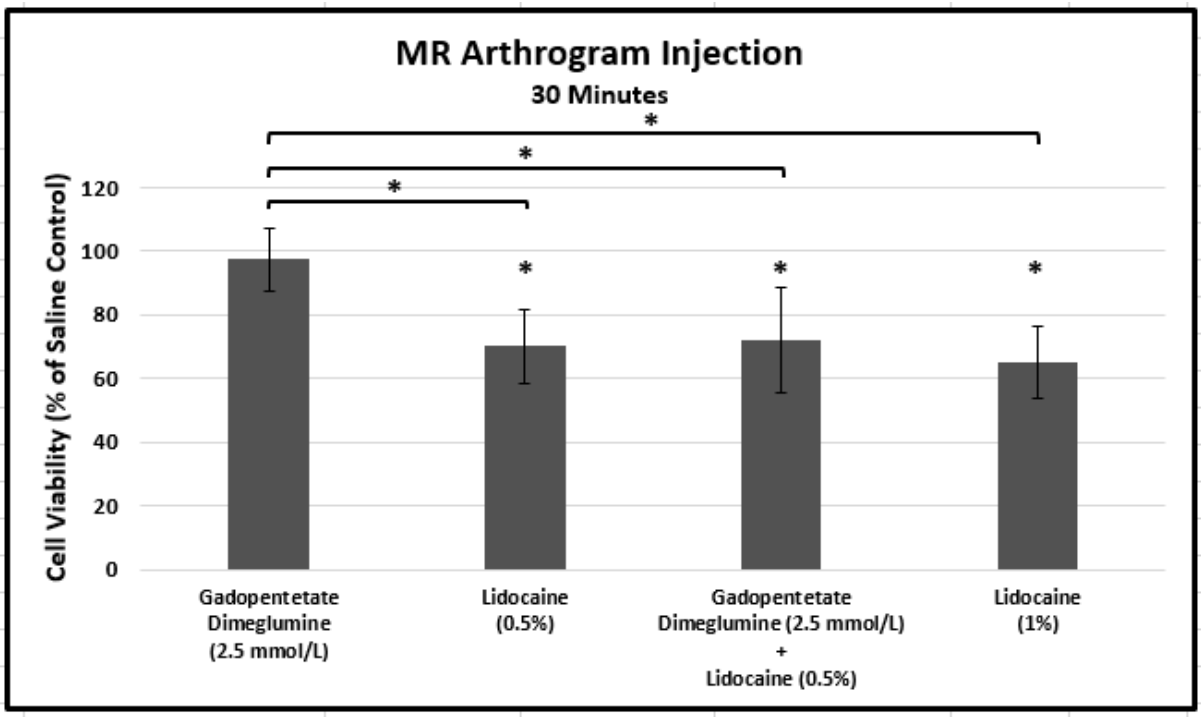

Figure 3.37 Bar graph illustrating the effect of treatment of individual agents, and combinations of agents, of an MR Arthrogram Clinical Solution for 30 minutes on cell viability of primary human chondrocytes using crystal violet staining. Data represents six independent experiments

* Statistically significant difference in cell viability from saline control

* Statistically significant inter-solution difference in cell viability

3.7.2.2 MR Arthrogram Clinical Solution: 45 Minutes

A Shapiro-Wilks test and Kolmogorov-Smirnov test ($p > 0.05$) and a visual inspection of their histograms, normal Q-Q plots and box plots showed that cell viability scores were approximately normally distributed for each solution, with a skewness of -0.12 (SE = 0.51) and a kurtosis of -0.73 (SE = 1.0) for Gadopentetate Dimeglumine (2.5 mmol/L), a skewness of 0.25 (SE = 0.44) and a kurtosis of -0.03 (SE = 0.95) for Lidocaine (0.5%), a skewness of 0.22 (SE = 0.43) and a kurtosis of 0.47 (SE = 0.83) for Gadopentetate Dimeglumine (2.5 mmol/L) + Lidocaine (0.5%) and a skewness of 0.69 (SE = 0.45) and a kurtosis of -0.44 (SE = 0.79) for Lidocaine (1%).

At 45 minutes there was no significant difference in cell viability between the saline control and Gadopentetate Dimeglumine (2.5 mmol/L) ($p = 1.0$; 95% CI, -15.5 – 17.4). There was a significant difference in cell viability between the saline control and Lidocaine (0.5%) ($p < 0.001$; 95% CI, 12.6 – 47.1), Gadopentetate Dimeglumine (2.5 mmol/L) + Lidocaine (0.5%) ($p <0.001$; 95% CI, 20.1 – 44.2) and Lidocaine 1% ($p < 0.001$; 95% CI, 26.0 – 58.3).

A significant inter-solution difference was found between Gadopentetate Dimeglumine (2.5 mmol/L) and each of Lidocaine 0.5% ($p < 0.001$; 95% CI, 20.2 – 51.7), Gadopentetate Dimeglumine (2.5 mmol/L) + Lidocaine (0.5%) ($p <0.001$; 95% CI, 20.4 – 52.5) and Lidocaine 1% ($p < 0.001$; 95% CI, 35.9 – 69.5).

The remaining inter-solution comparisons did not reach significance:

- Lidocaine 0.5% - Gadopentetate Dimeglumine (2.5 mmol/L) + Lidocaine (0.5%) ($p = 1.0$; 95% CI, -14.4 – 15.8).
- Lidocaine 0.5 % - Lidocaine 1% ($p = 0.69$; 95% CI, -7.8 – 22.6).
- Gadopentetate Dimeglumine (2.5 mmol/L) + Lidocaine (0.5%) – Lidocaine 1% ($p = 0.36$; 95% CI, -7.8 – 21.9).

Table 3.38 Effect of treatment of individual agents, and combinations of agents, of an MR Arthrogram Clinical Solution for 45 minutes on cell viability of primary human chondrocytes using crystal violet staining. Data represents six independent experiments

	Gadopentetate Dimeglumine (2.5 mmol/L)	**Lidocaine (0.5%)**	**Gadopentetate Dimeglumine (2.5 mmol/L) + Lidocaine (0.5%)**	**Lidocaine (1%)**	***p*-value**
Mean	99.9	68.9	67.0	57.9	
95% CI	93.0 – 105.2	60.1 – 78.8	59.4 – 79.8	52.0 – 67.2	
SD	5.4	7.7	6.4	11.2	
Range	92.8 – 110.1	58.4 – 85.6	54.5 – 83.7	36.3 – 71.8	
					$p < 0.001$

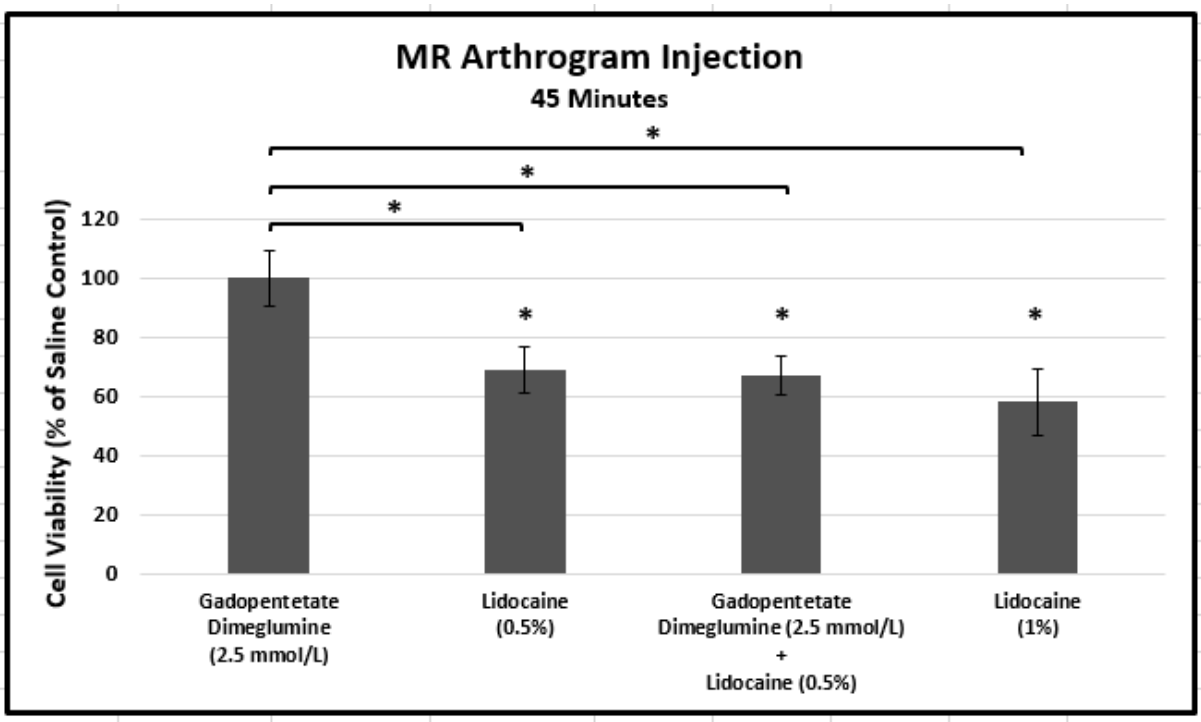

Figure 3.38 Bar graph illustrating the effect of treatment of individual agents, and combinations of agents, of an MR Arthrogram Clinical Solution for 45 minutes on cell viability of primary human chondrocytes using crystal violet staining. Data represents six independent experiments

* Statistically significant difference in cell viability from saline control

* Statistically significant inter-solution difference in cell viability

3.7.2.3 MR Arthrogram Clinical Solution: 60 Minutes

A Shapiro-Wilks test and Kolmogorov-Smirnov test ($p > 0.05$) and a visual inspection of their histograms, normal Q-Q plots and box plots showed that cell viability scores were approximately normally distributed for each solution, with a skewness of 0.34 (SE = 1.23) and a kurtosis of 0.45 (SE = 2.7) for Gadopentetate Dimeglumine (2.5 mmol/L), a skewness of 1.18 (SE = 1.01) and a kurtosis of 0.43 (SE = 2.6) for Lidocaine (0.5%), a skewness of -1.17 (SE = 1.01) and a kurtosis of 1.94 (SE = 2.62) for Gadopentetate Dimeglumine (2.5 mmol/L) + Lidocaine (0.5%) and a skewness of -1.14 (SE = 1.01) and a kurtosis of 0.59 (SE = 2.62) for Lidocaine (1%).

At 60 minutes there was no significant difference in cell viability between the saline control and Gadopentetate Dimeglumine (2.5 mmol/L) ($p = 1.0$; 95% CI -7.9 – 19.1). There was a significant difference in cell viability between the saline control and Lidocaine (0.5%) ($p < 0.001$; 95% CI, 20.7 – 49.5), Gadopentetate Dimeglumine (2.5 mmol/L) + Lidocaine (0.5%) ($p <0.001$; 95% CI, 19.6 – 48.3) and Lidocaine 1% ($p < 0.001$; 95% CI, 43.7 – 73.9).

A significant inter-solution difference was found between Gadopentetate Dimeglumine (2.5 mmol/L) and each of Lidocaine 0.5% ($p < 0.001$; 95% CI, 14.8 – 44.2), Gadopentetate Dimeglumine (2.5 mmol/L) + Lidocaine (0.5%) ($p <0.001$; 95% CI, 13.6 – 43.1) and Lidocaine 1% ($p < 0.001$; 95% CI, 37.7 – 68.6).

A significant inter-solution difference was also found between Lidocaine 0.5 % and Lidocaine 1% ($p < 0.001$; 95% CI, 7.5 – 39.9) and between Gadopentetate Dimeglumine (2.5 mmol/L) + Lidocaine (0.5%) and Lidocaine 1% ($p < 0.001$; 95% CI, -41.0 - -8.6).

The remaining inter-solution comparison did not reach significance:

- Lidocaine 0.5% - Gadopentetate Dimeglumine (2.5 mmol/L) + Lidocaine (0.5%) ($p = 1.0$; 95% CI, -16.7 – 14.4).

Table 3.39 Effect of treatment of individual agents, and combinations of agents, of an MR Arthrogram Clinical Solution for 60 minutes on cell viability of primary human chondrocytes using crystal violet staining. Data represents six independent experiments

	Gadopentetate Dimeglumine (2.5 mmol/L)	**Lidocaine (0.5%)**	**Gadopentetate Dimeglumine (2.5 mmol/L) + Lidocaine (0.5%)**	**Lidocaine (1%)**	***p*-value**
Mean	95.6	63.4	65.1	41.6	
95% CI	73.4 – 118.2	41.0 – 85.8	42.7 – 87.4	18.2 – 65.1	
SD	9.03	14.1	14.1	14.7	
Range	87.1 – 105.1	52.3 – 82.8	45.5 – 78.6	21.4 – 54.4	
					$p < 0.001$

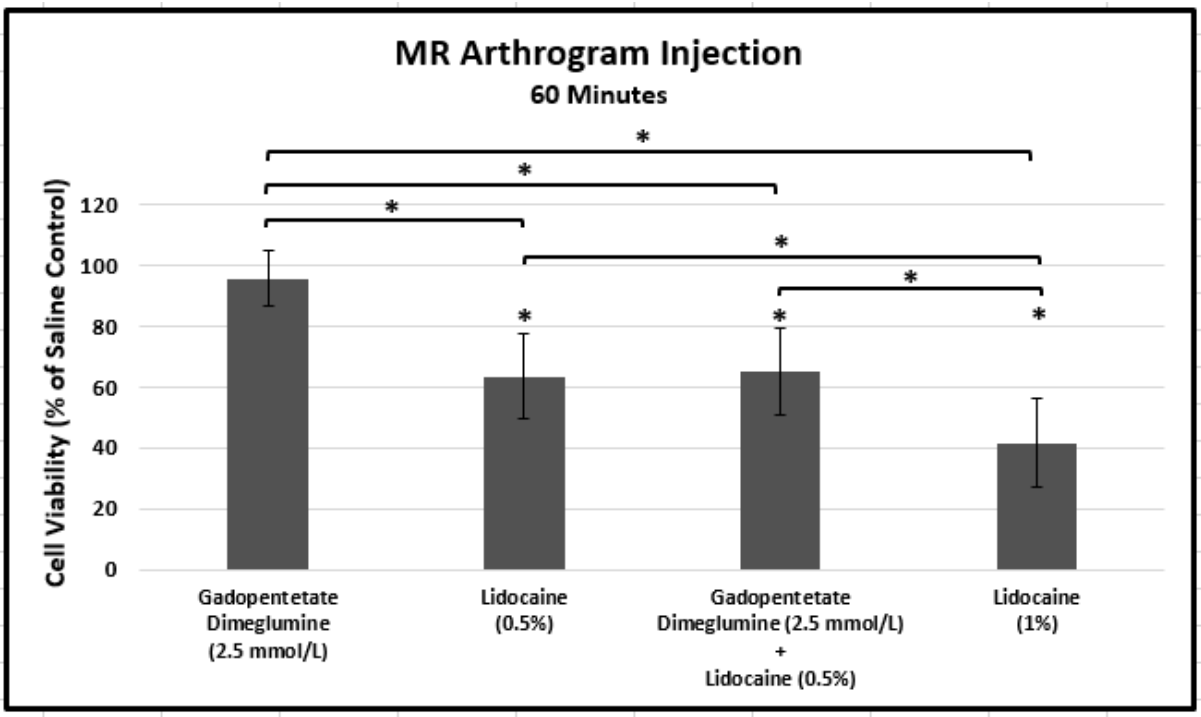

Figure 3.39 Bar graph illustrating the effect of treatment of individual agents, and combinations of agents, of an MR Arthrogram Clinical Solution for 60 minutes on cell viability of primary human chondrocytes using crystal violet staining. Data represents six independent experiments

* Statistically significant difference in cell viability from saline control

* Statistically significant inter-solution difference in cell viability

3.8 The effect of a Conventional / CT Arthrogram Clinical Solution on primary human chondrocytes

To investigate the effect of individual agents, and combinations of agents, of either a Conventional or CT Arthrogram Clinical Solution on primary human chondrocyte cell viability, cells were treated with varying agents, either alone or in combination with other agents [either Iohexol (150 mg/ml), Lidocaine (0.5%), Iohexol (150 mg/ml) + Lidocaine (0.5%) or Lidocaine (1%)] for 30 minutes, 45 minutes and 60 minutes (Section 2.3.5.4). Shorter time periods were selected as solutions were made without cell media. Cells exposed to normal saline served as the controls. Cell viability was then assessed using the MTS assay (Section 2.3.6.1) and crystal violet staining (Section 2.3.6.2). All data represents the results of six independent experiments.

3.8.1 Conventional / CT Arthrogram Clinical Solution: The effect of individual agents, and combinations of agents, on primary human chondrocyte viability using an MTS assay

3.8.1.1 Conventional / CT Arthrogram Clinical Solution: 30 Minutes

A Shapiro-Wilks test and Kolmogorov-Smirnov test ($p > 0.05$) and a visual inspection of their histograms, normal Q-Q plots and box plots showed that cell viability scores were approximately normally distributed for each solution, with a skewness of 1.12 (SE = 0.45) and a kurtosis of -0.04 (SE = 0.87) for Iohexol (150 mg/ml), a skewness of 0.18 (SE = 0.45) and a kurtosis of -0.42 (SE = 0.87) for Lidocaine (0.5%), a skewness of 0.84 (SE = 0.49) and a kurtosis of -0.28 (SE = 0.95) for Iohexol (150 mg/ml)+ Lidocaine (0.5%) and a skewness of 0.49 (SE = 0.47) and a kurtosis of -0.62 (SE = 0.92) for Lidocaine (1%).

At 30 minutes there was no significant difference in cell viability between the saline control and Iohexol (150 mg/ml) ($p = 1.0$; 95% CI, -15.3 – 21.8). There was a significant difference in cell viability between the saline control and Lidocaine (0.5%) ($p < 0.001$; 95% CI, 12.3 – 48.6), Iohexol (150 mg/ml) + Lidocaine (0.5%) ($p <0.001$; 95% CI, 9.2 – 47.6) and Lidocaine (1%) ($p < 0.001$; 95% CI, 16.1 – 53.7).

A significant inter-solution difference was found between Iohexol (150 mg/ml) and each of Lidocaine (0.5%) ($p = 0.001$; 95% CI, 8.6 – 45.7), Iohexol (150 mg/ml) + Lidocaine (0.5%) ($p = 0.004$; 95% CI, 5.6 – 44.6) and Lidocaine (1%) ($p < 0.001$; 95% CI, 12.6 – 50.7).

The remaining inter-solution comparisons did not reach significance:

- Lidocaine (0.5%) – Iohexol (150 mg/ml) + Lidocaine (0.5%) (p = 1.0; 95% CI, -17.1 – 21.3).
- Lidocaine (0.5 %) – Lidocaine (1%) (p = 1.0; 95% CI, -14.2 – 23.2).
- Iohexol (150 mg/ml) + Lidocaine (0.5%) – Lidocaine (1%) (p = 1.0; 95% CI, -13.2 – 26.3)

Table 3.40 Effect of treatment of individual agents, and combinations of agents, of a Conventional or CT Arthrogram Clinical Solution for 30 minutes on cell viability of primary human chondrocytes using the MTS assay. Data represents six independent experiments

	Iohexol (150 mg/ml)	**Lidocaine (0.5%)**	**Iohexol (150 mg/ml) + Lidocaine (0.5%)**	**Lidocaine (1%)**	***p*-value**
Mean	96.7	69.6	71.6	65.1	
95% CI	84.4 – 109.0	60.3 – 78.8	58.3 – 84.9	55.9 – 74.3	
SD	14.9	11.7	14.9	10.9	
Range	73.2 – 113.6	49.5 – 97.4	36.2 – 82.6	34.6 – 80.0	
					$p < 0.001$

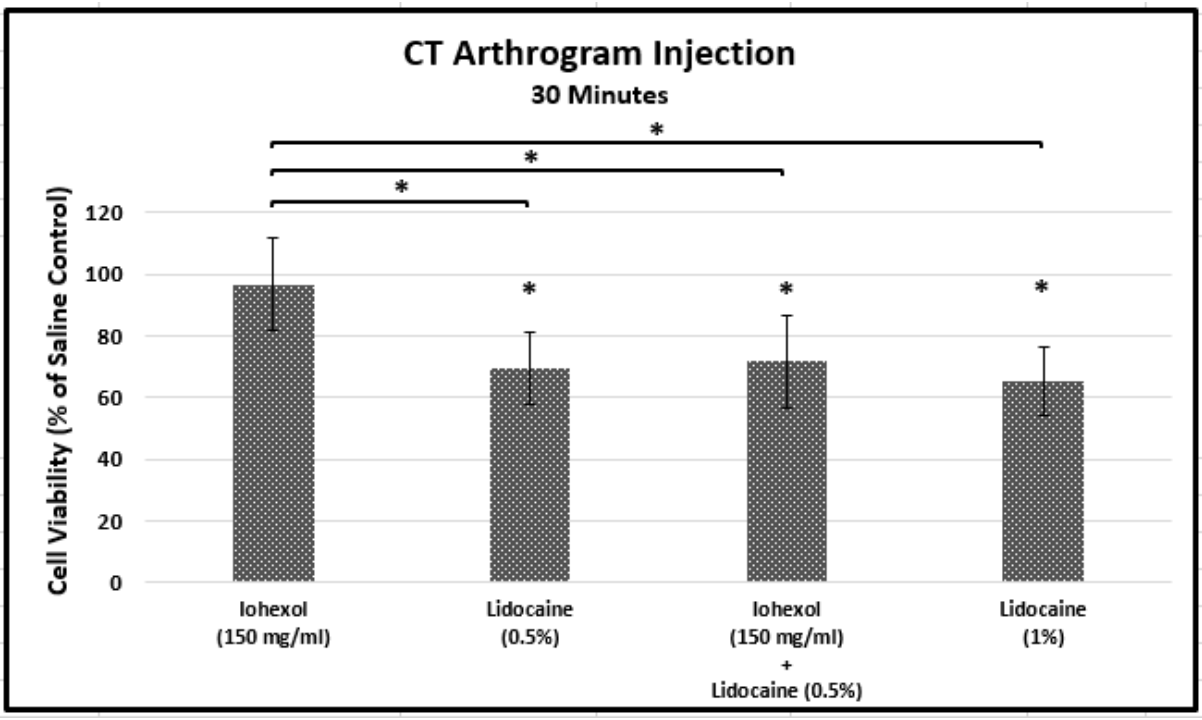

Figure 3.40 Bar graph illustrating the effect of treatment of individual agents, and combinations of agents, of a Conventional or CT Arthrogram Clinical Solution for 30 minutes on cell viability of primary human chondrocytes using the MTS assay. Data represents six independent experiments

* Statistically significant difference in cell viability from saline control

* Statistically significant inter-solution difference in cell viability

3.8.1.2 Conventional / CT Arthrogram Clinical Solution: 45 Minutes

A Shapiro-Wilks test and Kolmogorov-Smirnov test ($p > 0.05$) and a visual inspection of their histograms, normal Q-Q plots and box plots showed that cell viability scores were approximately normally distributed for each solution, with a skewness of -0.13 (SE = 0.52) and a kurtosis of -0.71 (SE = 1.01) for Iohexol (150 mg/ml), a skewness of 0.20 (SE = 0.49) and a kurtosis of -0.04 (SE = 0.95) for Lidocaine (0.5%), a skewness of 0.22 (SE = 0.43) and a kurtosis of 0.47 (SE = 0.83) for Iohexol (150 mg/ml)+ Lidocaine (0.5%) and a skewness of 0.71 (SE = 0.42) and a kurtosis of -0.41 (SE = 0.83) for Lidocaine (1%).

At 45 minutes there was no significant difference in cell viability between the saline control and Iohexol (150 mg/ml) ($p = 1.0$; 95% CI, -16.5 – 18.6). There was a significant difference in cell viability between the saline control and Lidocaine (0.5%) ($p < 0.001$; 95% CI, 24.4 – 58.1), Iohexol (150 mg/ml) + Lidocaine (0.5%) ($p <0.001$; 95% CI, 25.0 – 56.2) and Lidocaine (1%) ($p < 0.001$; 95% CI, 36.0 – 67.3).

A significant inter-solution difference was found between Iohexol (150 mg/ml) and each of Lidocaine (0.5%) ($p < 0.001$; 95% CI, 22.2 – 58.3), Iohexol (150 mg/ml) + Lidocaine (0.5%) ($p < 0.001$; 95% CI, 22.7 – 56.5) and Lidocaine (1%) ($p < 0.001$; 95% CI, 33.7 – 67.5).

The remaining inter-solution comparisons did not reach significance:

- Lidocaine (0.5%) – {Iohexol (150 mg/ml) + Lidocaine (0.5%)} ($p = 1.0$; 95% CI, -15.5 – 16.8).
- Lidocaine (0.5 %) – Lidocaine (1%) ($p = 0.69$; 95% CI, -5.8 – 26.6).
- Iohexol (150 mg/ml) + Lidocaine (0.5%) – Lidocaine (1%) ($p = 0.36$; 95% CI, -3.8 – 25.9)

Table 3.41 Effect of treatment of individual agents, and combinations of agents, of a Conventional or CT Arthrogram Clinical Solution for 45 minutes on cell viability of primary human chondrocytes using the MTS assay. Data represents six independent experiments

	Iohexol (150 mg/ml)	**Lidocaine (0.5%)**	**Iohexol (150 mg/ml) + Lidocaine (0.5%)**	**Lidocaine (1%)**	***p*-value**
Mean	98.9	58.7	59.4	48.3	
95% CI	87.8 – 110.2	50.5 – 67.0	51.5 – 67.3	38.7 – 57.9	
SD	11.6	9.3	10.5	12.9	
Range	70.0 – 122.9	30.4 – 81.2	29.2 – 84.4	11.2 – 70.9	
					$p < 0.001$

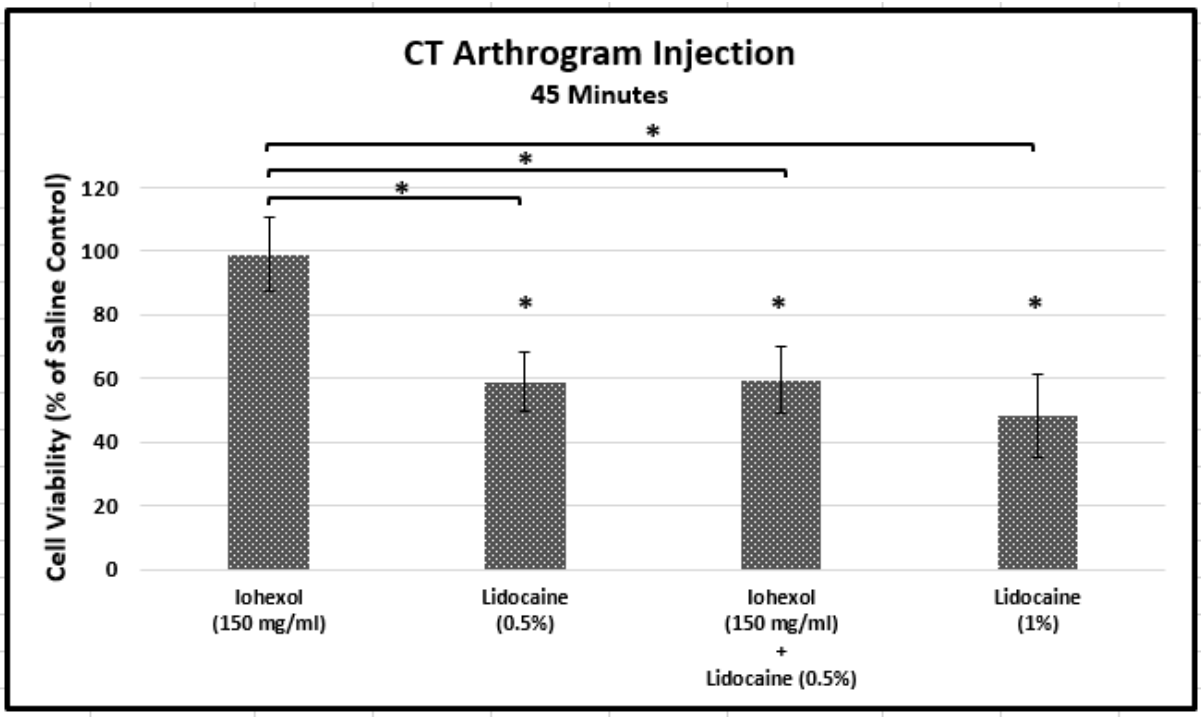

Figure 3.41 Bar graph illustrating the effect of treatment of individual agents, and combinations of agents, of a Conventional or CT Arthrogram Clinical Solution for 45 minutes on cell viability of primary human chondrocytes using the MTS assay. Data represents six independent experiments

* Statistically significant difference in cell viability from saline control

* Statistically significant inter-solution difference in cell viability

3.8.1.3 Conventional / CT Arthrogram Clinical Solution: 60 Minutes

A Shapiro-Wilks test and Kolmogorov-Smirnov test ($p > 0.05$) and a visual inspection of their histograms, normal Q-Q plots and box plots showed that cell viability scores were not normally distributed for each solution, with a skewness of 0.98 (SE = 0.46) and a kurtosis of -1.11 (SE = 0.89) for Iohexol (150 mg/ml), a skewness of 0.76 (SE = 0.44) and a kurtosis of 0.47 (SE = 0.86) for Lidocaine (0.5%), a skewness of 0.94 (SE = 0.43) and a kurtosis of 0.24 (SE = 0.85) for Iohexol (150 mg/ml)+ Lidocaine (0.5%) and a skewness of 0.96 (SE = 0.39) and a kurtosis of 0.13 (SE = 0.77) for Lidocaine (1%).

At 60 minutes there was no significant difference in cell viability between the saline control and Iohexol (150 mg/ml) ($p = 1.0$; SE 11.6). There was a significant difference in cell viability between the saline control and Lidocaine (0.5%) ($p < 0.001$; SE 11.4), Iohexol (150 mg/ml) + Lidocaine (0.5%) ($p <0.001$; SE 11.3) and Lidocaine (1%) ($p < 0.001$; SE 10.7).

A significant inter-solution difference was found between Iohexol (150 mg/ml) and each of Lidocaine (0.5%) ($p = 0.002$; SE 11.7), Iohexol (150 mg/ml) + Lidocaine (0.5%) ($p < 0.001$; SE 11.6) and Lidocaine (1%) ($p < 0.001$; SE 11.0).

The remaining inter-solution comparisons did not reach significance:

- Lidocaine (0.5%) – Iohexol (150 mg/ml) + Lidocaine (0.5%) ($p = 1.0$; SE 11.4).
- Lidocaine (0.5 %) – Lidocaine (1%) ($p = 0.2$; SE 10.8).
- Iohexol (150 mg/ml) + Lidocaine (0.5%) – Lidocaine (1%) ($p = 0.48$; SE 10.7)

Table 3.42 Effect of treatment of individual agents, and combinations of agents, of a Conventional or CT Arthrogram Clinical Solution for 60 minutes on cell viability of primary human chondrocytes using the MTS assay. Data represents six independent experiments

	Iohexol (150 mg/ml)	**Lidocaine (0.5%)**	**Iohexol (150 mg/ml) + Lidocaine (0.5%)**	**Lidocaine (1%)**	***p*-value**
Mean	96.5	58.8	54.3	40.5	
95% CI	84.3 – 108.6	48.9 – 68.7	43.8 – 64.8	33.4 – 47.6	
SD	15.0	12.8	13.8	10.5	
Range	70.1 – 125.2	36.2 – 88.6	37.3 – 82.4	26.9 – 81.8	
					$p < 0.001$

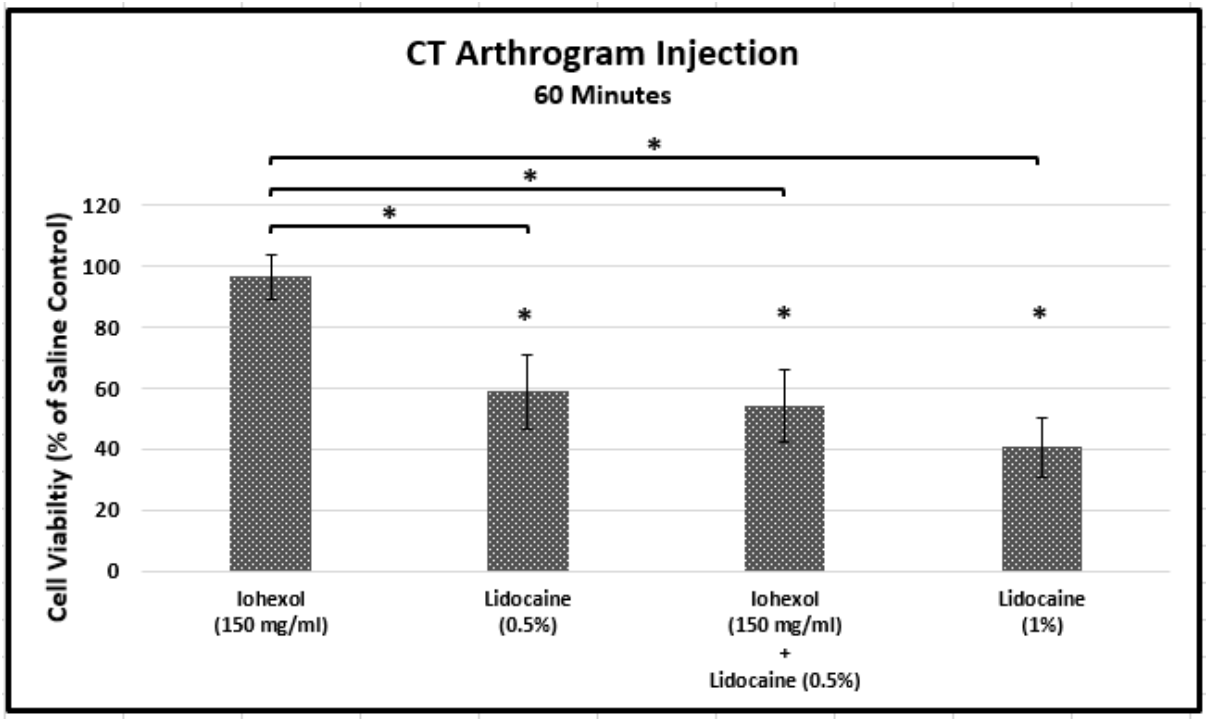

Figure 3.42 Bar graph illustrating the effect of treatment of individual agents, and combinations of agents, of a Conventional or CT Arthrogram Clinical Solution for 60 minutes on cell viability of primary human chondrocytes using the MTS assay. Data represents six independent experiments

* Statistically significant difference in cell viability from saline control

* Statistically significant inter-solution difference in cell viability

3.8.2 Conventional/ CT Arthrogram Clinical Solution: The effect of individual agents, and combinations of agents, on primary human chondrocyte viability using crystal violet staining.

3.8.2.1 Conventional/ CT Arthrogram Clinical Solution: 30 Minutes

A Shapiro-Wilks test and Kolmogorov-Smirnov test ($p > 0.05$) and a visual inspection of their histograms, normal Q-Q plots and box plots showed that cell viability scores were not normally distributed for each solution, with a skewness of 0.74 (SE = 0.64) and a kurtosis of -0.64 (SE = 1.23) for Iohexol (150 mg/ml), a skewness of -0.14 (SE = 0.64) and a kurtosis of -0.27 (SE = 1.23) for Lidocaine (0.5%), a skewness of 0.1 (SE = 0.66) and a kurtosis of 0.29 (SE = 1.28) for Iohexol (150 mg/ml)+ Lidocaine (0.5%) and a skewness of 0.57 (SE = 0.64) and a kurtosis of -1.65 (SE = 1.23) for Lidocaine (1%).

At 30 minutes there was no significant difference in cell viability between the saline control and Iohexol (150 mg/ml) ($p = 1.0$; SE 6.9). There was a significant difference in cell viability between the saline control and Lidocaine (0.5%) ($p < 0.001$; SE 6.9), Iohexol (150 mg/ml) + Lidocaine (0.5%) ($p <0.001$; SE 7.1) and Lidocaine (1%) ($p < 0.001$; SE 6.9).

A significant inter-solution difference was found between Iohexol (150 mg/ml) and each of Lidocaine (0.5%) ($p = 0.04$; SE 6.9), Iohexol (150 mg/ml) + Lidocaine (0.5%) ($p = 0.03$; SE 7.1) and Lidocaine (1%) ($p = 0.004$; SE 6.7).

The remaining inter-solution comparisons did not reach significance:

- Lidocaine (0.5%) – {Iohexol (150 mg/ml) + Lidocaine (0.5%)} ($p = 1.0$; SE 7.1).
- Lidocaine (0.5 %) – Lidocaine (1%) ($p = 1.0$; SE 6.9).
- Iohexol (150 mg/ml) + Lidocaine (0.5%) – Lidocaine (1%) ($p = 1.0$; SE 7.1)

Table 3.43 Effect of treatment of individual agents, and combinations of agents, of a Conventional or CT Arthrogram Clinical Solution for 30 minutes on cell viability of primary human chondrocytes using crystal violet staining. Data represents six independent experiments

	Iohexol (150 mg/ml)	**Lidocaine (0.5%)**	**Iohexol (150 mg/ml) + Lidocaine (0.5%)**	**Lidocaine (1%)**	***p*-value**
Mean	96.4	81.4	80.1	79.7	
95% CI	91.9 – 100.7	77.1 – 85.7	72.7 – 87.6	74.6 – 84.7	
SD	6.9	6.8	11.1	7.9	
Range	89.4 – 108.3	71.0 – 92.3	63.1 – 97.9	72.0 – 90.1	
					$p < 0.001$

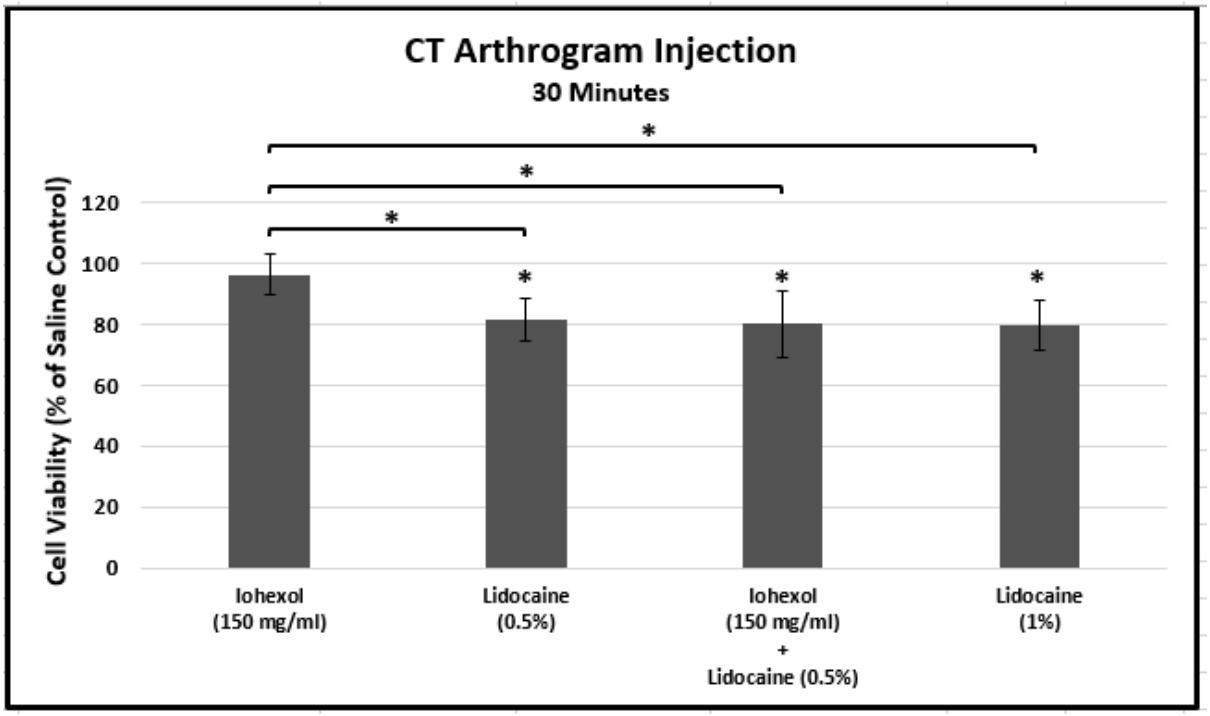

Figure 3.43 Bar graph illustrating the effect of treatment of individual agents, and combinations of agents, of a Conventional or CT Arthrogram Clinical Solution for 30 minutes on cell viability of primary human chondrocytes using crystal violet staining. Data represents six independent experiments

* Statistically significant difference in cell viability from saline control

* Statistically significant inter-solution difference in cell viability

3.8.2.2 Conventional/ CT Arthrogram Clinical Solution: 45 Minutes

A Shapiro-Wilks test and Kolmogorov-Smirnov test ($p > 0.05$) and a visual inspection of their histograms, normal Q-Q plots and box plots showed that cell viability scores were approximately normally distributed for each solution, with a skewness of -0.22 (SE = 0.51) and a kurtosis of -1.09 (SE = 0.99) for Iohexol (150 mg/ml), a skewness of -0.68 (SE = 0.52) and a kurtosis of -0.3 (SE = 1.01) for Lidocaine (0.5%), a skewness of -0.93 (SE = 0.47) and a kurtosis of 0.51 (SE = 0.92) for Iohexol (150 mg/ml)+ Lidocaine (0.5%) and a skewness of -0.33 (SE = 0.66) and a kurtosis of -0.27 (SE = 1.28) for Lidocaine (1%).

At 45 minutes there was no significant difference in cell viability between the saline control and Iohexol (150 mg/ml) ($p = 1.0$; 95% CI, -5.6 – 13.5). There was a significant difference in cell viability between the saline control and Lidocaine (0.5%) ($p < 0.001$; 95% CI, 15.2 – 34.5), Iohexol (150 mg/ml) + Lidocaine (0.5%) ($p <0.001$; 95% CI, 17.7 – 35.9) and Lidocaine (1%) ($p < 0.001$; 95% CI, 26.6 – 45.3).

A significant inter-solution difference was found between Iohexol (150 mg/ml) and each of Lidocaine (0.5%) ($p < 0.001$; 95% CI, 11.2 – 30.6), Iohexol (150 mg/ml) + Lidocaine (0.5%) ($p < 0.001$; 95% CI, 13.7 – 32.0) and Lidocaine (1%) ($p < 0.001$; 95% CI, 22.7 – 41.3).

A significant inter-solution difference was also found between Lidocaine (0.5%) and Lidocaine (1%) ($p = 0.01$; 95% CI, 1.2 – 20.6) and between Iohexol (150 mg/ml) + Lidocaine (0.5%) and Lidocaine (1%) ($p = 0.04$; 95% CI, 0.2 – 18.0).

The remaining inter-solution comparison did not reach significance:

- Lidocaine (0.5%) – Iohexol (150 mg/ml) + Lidocaine (0.5%) ($p = 1.0$; 95% CI, -7.3 – 11.3).

Table 3.44 Effect of treatment of individual agents, and combinations of agents, of a Conventional or CT Arthrogram Clinical Solution for 45 minutes on cell viability of primary human chondrocytes using crystal violet staining. Data represents six independent experiments

	Iohexol (150 mg/ml)	**Lidocaine (0.5%)**	**Iohexol (150 mg/ml) + Lidocaine (0.5%)**	**Lidocaine (1%)**	***p*-value**
Mean	96.0	75.2	73.2	64.0	
95% CI	90.4 – 101.7	69.7 – 80.6	68.5 – 77.8	55.5 – 72.6	
SD	12.1	11.2	10.9	12.7	
Range	73.7 – 117.0	50.3 – 88.4	45.5 – 86.8	40.6 – 84.5	
					$p < 0.001$

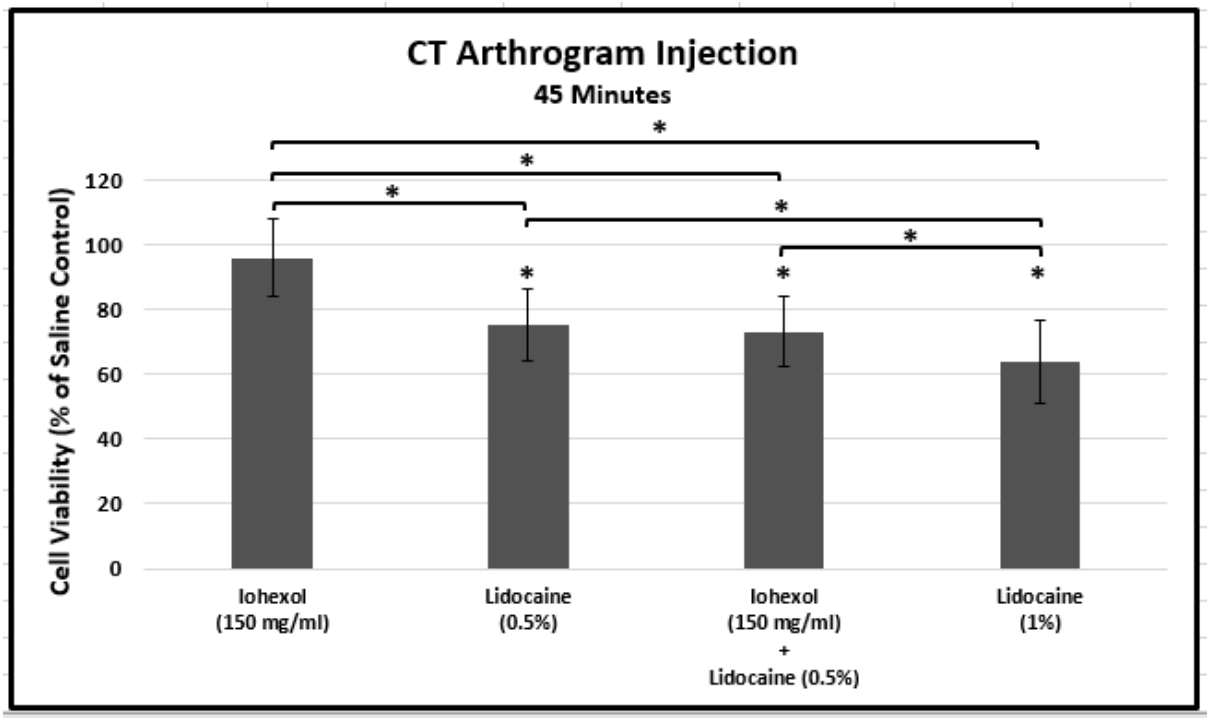

Figure 3.44 Bar graph illustrating the effect of treatment of individual agents, and combinations of agents, of a Conventional or CT Arthrogram Clinical Solution for 45 minutes on cell viability of primary human chondrocytes using crystal violet staining. Data represents six independent experiments

* Statistically significant difference in cell viability from saline control

* Statistically significant inter-solution difference in cell viability

3.8.2.3 Conventional/ CT Arthrogram Clinical Solution: 60 Minutes

A Shapiro-Wilks test and Kolmogorov-Smirnov test ($p > 0.05$) and a visual inspection of their histograms, normal Q-Q plots and box plots showed that cell viability scores were approximately normally distributed for each solution, with a skewness of -0.55 (SE = 0.55) and a kurtosis of 0.23 (SE = 1.06) for Iohexol (150 mg/ml), a skewness of -0.16 (SE = 0.44) and a kurtosis of -0.54 (SE = 0.86) for Lidocaine (0.5%), a skewness of 0.39 (SE = 0.46) and a kurtosis of 0.44 (SE = 0.89) for Iohexol (150 mg/ml)+ Lidocaine (0.5%) and a skewness of 0.34 (SE = 0.43) and a kurtosis of -1.47 (SE = 0.83) for Lidocaine (1%).

At 60 minutes there was no significant difference in cell viability between the saline control and Iohexol (150 mg/ml) ($p = 1.0$; 95% CI, -4.5 – 13.8). There was a significant difference in cell viability between the saline control and Lidocaine (0.5%) ($p < 0.001$; 95% CI, 18.4 – 34.3), Iohexol (150 mg/ml) + Lidocaine (0.5%) ($p <0.001$; 95% CI, 22.1 – 38.3) and Lidocaine (1%) ($p < 0.001$; 95% CI, 30.4 – 46.1).

A significant inter-solution difference was found between Iohexol (150 mg/ml) and each of Lidocaine (0.5%) ($p < 0.001$; 95% CI, 12.5 – 30.9), Iohexol (150 mg/ml) + Lidocaine (0.5%) ($p < 0.001$; 95% CI, 16.2 – 34.9) and Lidocaine (1%) ($p < 0.001$; 95% CI, 24.5 – 42.7).

A significant inter-solution difference was also found between Lidocaine (0.5%) and Lidocaine (1%) ($p < 0.001$; 95% CI, 4.0 – 19.8) and between Iohexol (150 mg/ml) + Lidocaine (0.5%) and Lidocaine (1%) ($p = 0.049$; 95% CI, 0.01 – 16.1).

The remaining inter-solution comparison did not reach significance:

- Lidocaine (0.5%) – Iohexol (150 mg/ml) + Lidocaine (0.5%) ($p = 1.0$; 95% CI, -4.0 – 12.0).

Table 3.45 Effect of treatment of individual agents, and combinations of agents, of a Conventional or CT Arthrogram Clinical Solution for 60 minutes on cell viability of primary human chondrocytes using crystal violet staining. Data represents six independent experiments

	Iohexol (150 mg/ml)	**Lidocaine (0.5%)**	**Iohexol (150 mg/ml) + Lidocaine (0.5%)**	**Lidocaine (1%)**	***p*-value**
Mean	95.4	73.6	69.8	61.7	
95% CI	91.0 – 99.7	68.4 – 78.9	63.6 – 76.0	58.7 – 64.7	
SD	8.4	13.5	15.4	8.0	
Range	76.2 – 108.2	46.4 – 99.9	41.8 – 108.7	52.3 – 75.4	
					$p < 0.001$

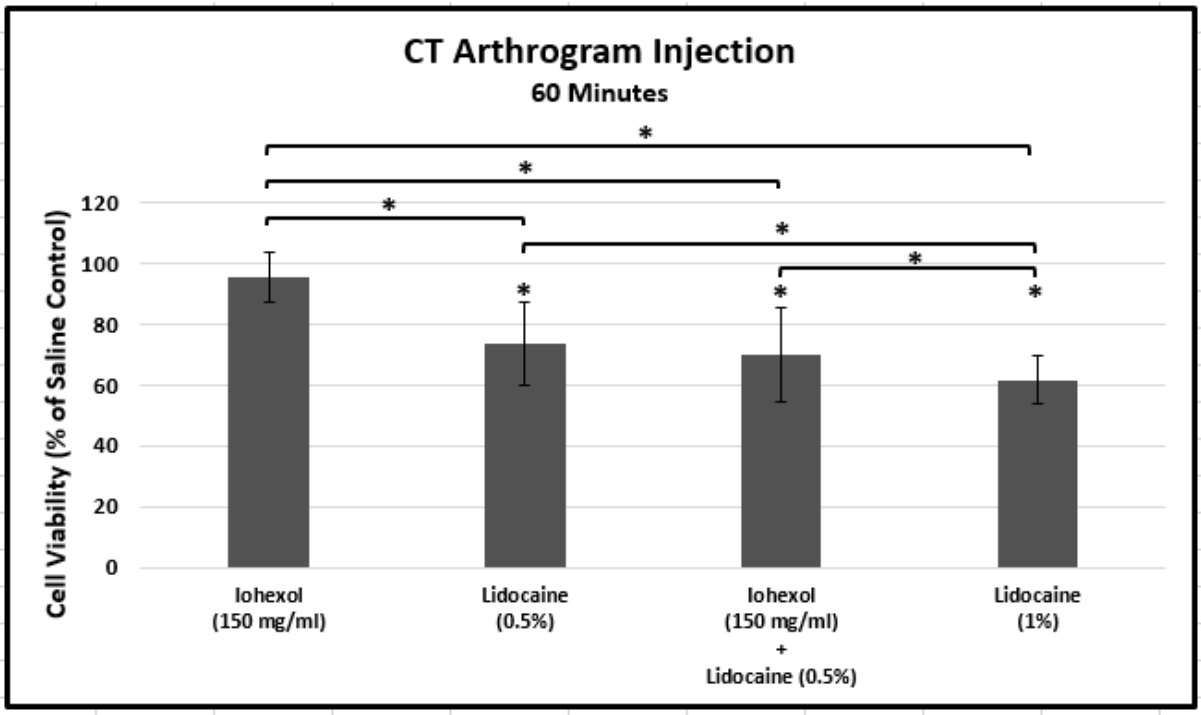

Figure 3.45 Bar graph illustrating the effect of treatment of individual agents, and combinations of agents, of a Conventional or CT Arthrogram Clinical Solution for 60 minutes on cell viability of primary human chondrocytes using crystal violet staining. Data represents six independent experiments

* Statistically significant difference in cell viability from saline control

* Statistically significant inter-solution difference in cell viability

3.9 The effect of Lidocaine on primary human chondrocytes

3.9.1 Concentration-dependent effect of Lidocaine on primary human chondrocytes

The concentration-dependent effect of Lidocaine on primary human chondrocytes is detailed in Sections 3.7 and 3.8.

3.9.2 Time-dependent effect of Lidocaine on primary human chondrocytes

3.9.2.1 Time-dependent effect of Lidocaine on primary human chondrocyte viability using the MTS assay

3.9.2.1.1 Lidocaine 0.5%

A Shapiro-Wilks test and Kolmogorov-Smirnov test ($p > 0.05$) and a visual inspection of their histograms, normal Q-Q plots and box plots showed that cell viability scores were approximately normally distributed for each time point, with a skewness of 0.55 (SE = 0.91) and a kurtosis of -1.4 (SE = 2.0) for 30 minutes, a skewness of 0.47 (SE = 1.0) and a kurtosis of -3.3 (SE = 2.6) for 45 minutes and a skewness of 0.39 (SE = 0.85) and a kurtosis of 1.4 (SE = 1.7) for 60 minutes.

There was a significant time-dependent effect of Lidocaine 0.5% on cell viability found between 30 minutes and both 45 minutes ($p = 0.016$; 95% CI, 2.5 – 28.5) and 60 minutes ($p = 0.003$; 95% CI, 5.1 – 28.6). There was no significant time-dependent effect between 45 minutes and 60 minutes ($p = 1.0$; 95% CI, -11.1 – 13.9).

Table 3.46 Effect of treatment with Lidocaine (0.5%) for varying time periods on cell viability of primary human chondrocytes using the MTS assay. Data represents six independent experiments.

	30 Minutes	**45 Minutes**	**60 Minutes**	***p*-value**
Mean	72.5	57.0	55.6	
95% CI	59.3 – 85.6	38.9 – 75.0	42.9 – 68.3	
SD	10.6	11.4	12.1	
Range	61.4 – 87.1	47.4 – 70.6	38.5 – 75.1	
				$p = 0.002$

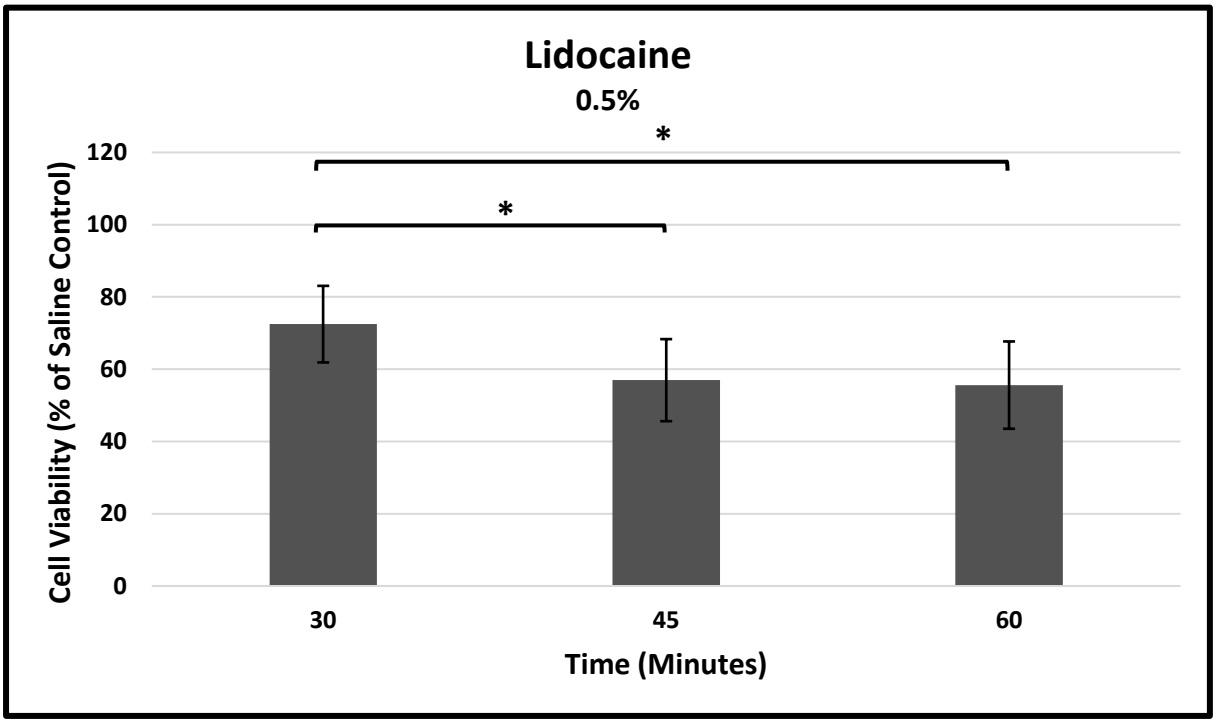

Figure 3.46 Bar graph illustrating the effect of treatment with Lidocaine (0.5%) for varying time periods on cell viability of primary human chondrocytes using the MTS assay. Data represents six independent experiments.

3.9.2.1.2 Lidocaine 1%

A Shapiro-Wilks test and Kolmogorov-Smirnov test ($p > 0.05$) and a visual inspection of their histograms, normal Q-Q plots and box plots showed that cell viability scores were not normally distributed for each time point, with a skewness of 0.05 (SE = 0.69) and a kurtosis of 0.08 (SE = 1.3) for 30 minutes, a skewness of 0.52 (SE = 0.72) and a kurtosis of -1.71 (SE = 1.4) for 45 minutes and a skewness of -1.13 (SE = 0.69) and a kurtosis of 0.44 (SE = 1.3) for 60 minutes.

There was a significant time-dependent effect of Lidocaine 1% on cell viability found between 30 minutes and both 45 minutes ($p = 0.006$, SE 3.9) and 60 minutes ($p = 0.006$, SE 3.8). There was no significant time-dependent effect between 45 minutes and 60 minutes ($p = 1.0$, SE 3.9).

Table 3.47 Effect of treatment with Lidocaine (1%) for varying time periods on cell viability of primary human chondrocytes using the MTS assay. Data represents six independent experiments.

	30 Minutes	**45 Minutes**	**60 Minutes**	***p*-value**
Mean	63.7	45.0	42.3	
95% CI	54.4 – 73.1	43.2 – 46.8	33.5 – 51.3	
SD	13.0	2.3	12.4	
Range	44.3 – 83.5	42.7 – 48.0	20.5 – 55.7	
				$p = 0.002$

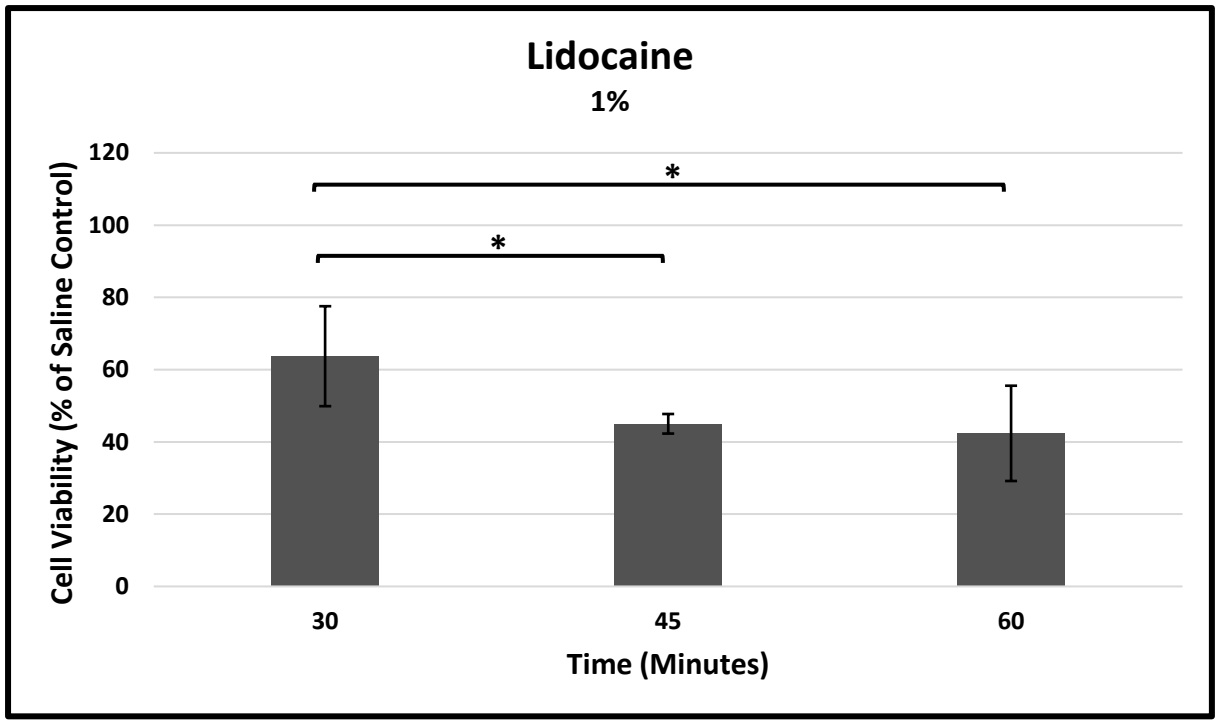

Figure 3.47 Bar graph illustrating the effect of treatment with Lidocaine (1%) for varying time periods on cell viability of primary human chondrocytes using the MTS assay. Data represents six independent experiments.

3.9.2.2 Time-dependent effect of Lidocaine on primary human chondrocyte viability using crystal violet staining

3.9.2.2.1 Lidocaine 0.5%

A Shapiro-Wilks test and Kolmogorov-Smirnov test ($p > 0.05$) and a visual inspection of their histograms, normal Q-Q plots and box plots showed that cell viability scores were approximately normally distributed for each time point, with a skewness of 0.19 (SE = 0.44) and a kurtosis of -0.32 (SE = 0.86) for 30 minutes, a skewness of -0.08 (SE = 0.38) and a kurtosis of -0.98 (SE = 0.74) for 45 minutes and a skewness of 0.01 (SE = 0.41) and a kurtosis of -1.23 (SE = 0.81) for 60 minutes.

There was a significant time-dependent effect of Lidocaine 0.5% on cell viability found between 30 minutes and both 45 minutes ($p = 0.008$; 95% CI, 3.1 – 26.3) and 60 minutes ($p = 0.009$; 95% CI, 3.1 – 27.4). There was no significant time-dependent effect between 45 minutes and 60 minutes ($p = 1.0$; 95% CI, -10.7 – 11.7).

Table 3.48 Effect of treatment with Lidocaine (0.5%) for varying time periods on cell viability of primary human chondrocytes using crystal violet staining. Data represents six independent experiments.

	30 Minutes	**45 Minutes**	**60 Minutes**	***p*-value**
Mean	79.5	68.8	63.4	
95% CI	60.6 – 80.4	49.6 – 70.0	47.3 – 71.2	
SD	11.9	16.0	9.7	
Range	47.7 – 92.4	24.3 – 85.8	42.5 – 75.1	
				$p = 0.002$

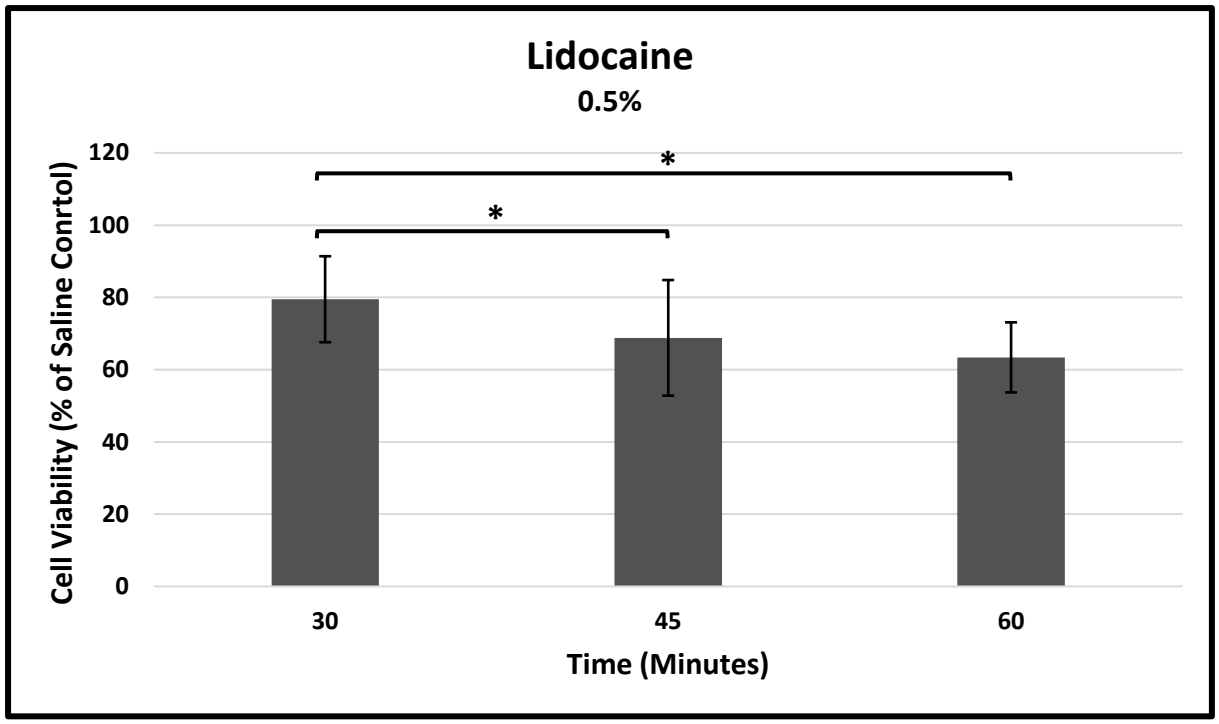

Figure 3.48 Bar graph illustrating the effect of treatment with Lidocaine (0.5%) for varying time periods on cell viability of primary human chondrocytes using crystal violet staining. Data represents six independent experiments.

3.9.2.2.2 Lidocaine 1%

A Shapiro-Wilks test and Kolmogorov-Smirnov test ($p > 0.05$) and a visual inspection of their histograms, normal Q-Q plots and box plots showed that cell viability scores were approximately normally distributed for each time point, with a skewness of -0.44 (SE = 0.46) and a kurtosis of -0.47 (SE = 0.91) for 30 minutes, a skewness of -0.22 (SE = 0.85) and a kurtosis of -1.09 (SE = 0.99) for 45 minutes and a skewness of 0.98 (SE = 0.46) and a kurtosis of -1.11 (SE = 0.89) for 60 minutes.

There was a significant time-dependent effect of Lidocaine 1% on cell viability found between 30 minutes and both 45 minutes ($p = 0.006$; 95% CI, 2.6 – 19.4) and 60 minutes ($p < 0.001$; 95% CI, 12.4 – 28.4). There was no significant time-dependent effect between 45 minutes and 60 minutes ($p = 0.06$; 95% CI, -0.14 – 18.5).

Table 3.49 Effect of treatment with Lidocaine (1%) for varying time periods on cell viability of primary human chondrocytes using crystal violet staining. Data represents six independent experiments

	30 Minutes	**45 Minutes**	**60 Minutes**	***p*-value**
Mean	72.0	61.0	51.6	
95% CI	66.9 – 77.1	55.4 – 73.6	46.1 – 57.2	
SD	12.3	9.7	10.4	
Range	44.3 – 90.1	45.6 – 84.5	41.6 – 61.7	
				$p < 0.001$

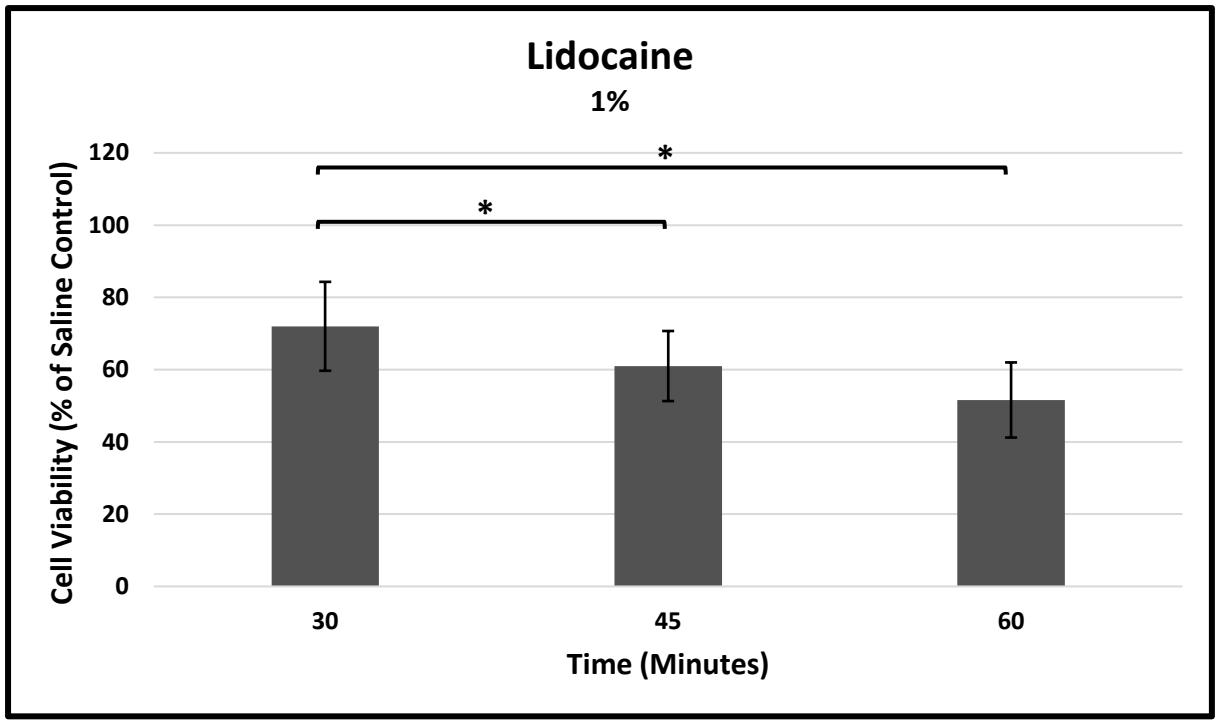

Figure 3.49 Effect of treatment with Lidocaine (1%) for varying time periods on cell viability of primary human chondrocytes using crystal violet staining. Data represents six independent experiments

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Chapter 4

Discussion

4.1 Isolation and cell culture of primary human chondrocytes

In order to examine the chondrotoxic effect of various intra-articular contrast agents and local anaesthetic, we isolated primary human chondrocytes from the femoral heads of patients undergoing bipolar hemiarthroplasty of the hip. Several protocols have been reported that describe chondrocyte cell culture and expansion from cartilage specimens harvested at the time of surgery (1-4). We aimed to find a protocol that would provide us with healthy cells in significant numbers in order to conduct our experiments.

Our removal of cartilage from the femoral heads did not differ significantly from other reports and similarly, we found that initial dicing of the cartilage aided in its subsequent digestion due to an increased surface area. However, during the digestion process of the cartilage chips, we did observe differences in chondrocyte health and appearance using three different protocols.

In our first protocol, we used 0.1% collagenase for 24 hours but found that the microscopic appearance of the cells was not satisfactory. Cartilage appeared to be under-digested and the majority of cells were still surrounded by matrix tissue. As a result, cells were unable to adhere to the flask surface and consequently, were unable to proliferate.

In our second protocol, we started with pronase 0.1% for 1 hour before subsequent digestion in 0.1% collagenase for 24 hours. The inclusion of pronase aided ECM digestion and we observed less debris and healthier cells than in our first protocol. Cells adhered to the flask surface more readily and were more confluent at Day 7 and Day 14. However, cell yields were significantly lower than with our first protocol.

In our third protocol, we increased the pronase concentration to 0.25% and decreased the collagenase concentration to 0.025%. We also reduced the collagenase digest time to 18 hours. This protocol resulted in phenotypically healthy chondrocytes with the highest cell yields. Therefore, our final protocol called for a 1 hour digestion with pronase 0.25%, followed by 18 hours digestion with collagenase 0.025%.

4.2 Chondrotoxicity of contrast agents

Despite their widespread use in musculoskeletal diagnosis, intra-articular contrast agents have received relatively little attention with regard to their effect on cartilage. An early study demonstrated that while free gadolinium is taken up completely by hyaline cartilage, gadopentetate dimeglumine (Gd-DTPA) bound to albumin diffuses much more slowly and only reaches the middle cartilage layers within 24 hours (5). However, it is unclear whether gadolinium chelates will complex with albumin *in vivo* and subsequent studies have shown that contrast agents diffuse freely through the matrix of cultured osteochondral explants of animals and humans (6-10). Penetration though articular cartilage is seen after both direct and indirect administration of contrast agents (6-10). In an *in vitro* study in which cartilage plugs harvested at TKA were immersed in 1 mmol/L ionic contrast for 90 minutes, tissue concentrations between 0.5 and 1 mmol/L were subsequently measured by magnetic resonance (9). The concentrations of non-ionic agents in cartilage are higher than ionic agents because they are not repelled by the negatively-charged proteoglycans. Therefore, it is likely that the concentration of non-ionic contrast agents in articular cartilage during direct arthrography is equivalent to their concentration in the injection solution (11).

Pharmacokinetics and clearance rates of intra-articular contrast rates are not completely understood. Apart from experimental results in animals (12, 13), only a few clinical studies exist which allow at least an estimation of the timeframe in which contrast agents leave the joint (7, 14-16). Gadolinium tetra-azacyclododecane tetraacetic acid (Gd-DOTA) at a concentration of 2 mmol/L has a half-life of approximately 2 hours in the synovial fluid of canine stifle joints (12). However, the half-life of gadolinium chelates in human joints may be considerably longer and some protocols include adrenaline in their solutions to prevent diffusion of contrast agent out of the joint (17). According to a study which examined the imaging window after intra-articular administration of Gd-DTPA into the human knee, penetration of gadolinium contrast into the patellar cartilage increased up to 8 hours after injection (7). In another study, Engel et al. (13) found very low concentrations of Gd-DTPA in histological sections of femoral cartilage between 17 and 137 hours after injection. However, no data was obtained prior to 17 hours. Precise intra-articular half-lives for iodinated contrast agents are similarly unknown, but clearance rates appear to be faster than gadolinium chelates. In the canine stifle joint, Katzberg et al. (18) found that 10 minutes after injection of dimer methylglucamine salts and metrizamide, less than 30% of the initial iodine concentration was still present. The resorption of a contrast agent over time, as a result of

diffusion through the synovial membrane and dilution, should slow down with increasing molecular weight and decreasing osmolarity (12, 19). Therefore, the longer intra-articular half-life of gadolinium chelates may be attributable to their lower osmolarity, which limits intra-articular fluid ingress. While 24 hours treatment with contrast agents exceeds the anticipated clinical exposure of articular chondrocytes during arthrography, we chose a time range of 1 to 24 hours to ensure our experiments were maximally sensitive to low levels of toxicity.

Systemic administration of contrast agents has largely been considered safe in adults and children (20). These studies have focused on acute toxicity and adverse reactions, and have determined safe systemic levels. However, detrimental effects are possible and toxicity has been demonstrated in other tissues. In a rat model, Gabelmann et al. (21) showed that intra-aortic injection of iodinated contrast agents at a concentration of 300 mg/ml lead to reversible transient focal endothelial cell injury. Meanwhile, gadolinium chelates have been found to cause arrhythmias and decrease inotropy in animal hearts *in vivo* and *in vitro* by interfering with electrical conduction (22, 23). Free gadolinium is extremely toxic. It is an inorganic blocker of many types of voltage gated calcium ion channels at nano- and micro-molar concentrations (24, 25) and inhibits physiological processes that depend on calcium influx. It may depress the reticuloendothelial system and has been shown to increase the expression of hepatic cytokines (20). Moreover, gadolinium can induce apoptosis in macrophages via a mechanism separate to the inhibition of calcium influx. Therefore, all available gadolinium-contrast agents are chelates of the gadolinium ion to reduce its toxicity. Cyclic chelates with high thermal stability constants and long dissociation half-lives are the least likely to release free gadolinium (26).

Relatively few studies have examined the potential toxicity of intra-articular contrast agents on chondrocytes or cartilage (11, 12, 19, 27-29). Furthermore, of the limited studies that have been performed, most were in animal models or used commercial cell lines, known to be at risk of losing their phenotypic or genotypic features (28). Two studies have considered the effect of iodinated contrast agents (28, 29) and only one has used human articular chondrocytes (28). Safety of intra-articular gadolinium was examined by Hajek et al. (19) and Rahmouni et al. (12), where solutions of 0.5 mmol/L Gd-DTPA or 50 mmol/L Gd-DOTA, respectively, were injected into animal knees. Gross inspection and light microscopy revealed no evidence of cartilage change and the authors concluded that gadolinium chelates are safe for intra-articular use. Although contrast agents have been found to cause adverse local

effects, these seem to occur almost exclusively at supra-therapeutic concentrations. Kose et al. (29) found evidence of histological and ultrastructural alterations in rabbit cartilage that failed to completely normalise within their observation period, yet this was after "high dose exposure" to either iopromide or gadopentetate dimeglumine. Similarly, *in vitro* studies have demonstrated cytotoxicity at doses far greater than those used in clinical practice. While Midura et al. (27) found little to no evidence of toxicity in a chondrocyte monolayer following 48-hour exposure to Gd-DTPA solutions up to 7.5 mmol/L, Oznam et al. (28) reported that metabolic activity and cell viability were significantly reduced following 6-hour exposure to Gd-DTPA at 20 mmol/L. However, as gadolinium formulations are typically diluted to a concentration of 1 to 2 mmol/L (30, 31), 20 mmol/L represents a minimum tenfold (x10) increase in concentration over clinical solutions. In similar fashion, Rahmouni et al. (12) showed that chondrocyte proliferation was only decreased by exposure to Gd-DOTA at a supra-therapeutic concentration of 50 mmol/L for 24 or 48 hours. In another study, Greisberg et al. (11) noted that although cell proliferation decreased following exposure to gadodiamide in a dose-dependent fashion, the only significant reduction occurred at a concentration of 40 mmol/L. Apoptosis was noted to increase across the test groups in a dose-dependent manner with 0.01% of cells in the 200 mmol/L group appearing apoptotic after 16 hours exposure. Although statistically significant, the authors question if such a small effect is clinically relevant, especially as 200 mmol/L represents a 100-fold increase in concentration over solutions used in direct arthrography. Beyond these studies, we are aware of no other work assessing the potential chondrotoxicity of intra-articular contrast agents.

We evaluated both iodinated- and gadolinium-based contrast agents as well as the chondrotoxicity of clinical solutions when mixed with local anaesthetic. In our first set of experiments, we investigated the concentration-dependent and time-dependent effects of iohexol and iopromide on cell viability by exposing primary human chondrocytes to clinically relevant solutions (either 50 mg/ml, 100 mg/ml or 200 mg/ml) for 1 hour, 4 hours, 16 hours or 24 hours. At 1 and 4 hours, across all concentrations, iodinated-contrast-treated cells yielded similar formazan staining patterns as media controls. However, following 16 hours exposure, a significant reduction in cell viability was noted at both 100 mg/ml and 200 mg/ml in the iohexol-treated group and at 200 mg/ml in the iopromide-treated group. At 24 hours, there was no difference from media controls at any concentration in the iohexol-treated group, but formazan content was significantly reduced at 100 mg/ml and 200 mg/ml in the iopromide-treated group. Expressed as a percentage of the media control, the lowest cell

viability (86.8% ± 2.6) was noted in cultures treated with iohexol at a concentration of 200 mg/ml for 16 hours. Although it may be possible that exposure to higher doses of iohexol for 16 hours initially suppressed metabolic activity, which then recovered by 24 hours, we believe our results more accurately reflect a decreasing volume of media used across the test solutions. This is readily seen in the iopromide-treated group, where a significant reduction at 16-hours exposure to 200 mg/ml is followed by further decreases at 24-hours exposure to both 100 mg/ml and 200 mg/ml. Each contrast agent was diluted in cell culture media in order to achieve its target concentration. In comparison to a 50 mg/ml solution of either iohexol or iopromide, there was 50% less media in the 100 mg/ml solutions and 75% less in the 200 mg/ml solutions. While no effect was seen at 1 and 4 hours, nutrient deprivation likely became a factor with longer exposure times (32). Furthermore, although we achieved statistical significance at 16 and 24 hours (each sub-experiment was repeated six times), it is doubtful whether such small effects in monolayer culture are clinically relevant. These results were mirrored in the time-dependent effects. While cultures treated with 100 mg/ml iohexol and iopromide had significant reductions in formazan staining patterns at 16 and 24 hours, respectively, when compared to both 1 and 4 hours exposure, no evidence of time-dependent effects were observed across 1, 4, 16 and 24 hours exposure to iohexol and iopromide at 50 mg/ml and 200 mg/ml.

In our second set of experiments, we investigated the concentration-dependent and time-dependent effects of gadopentetate dimeglumine and gadobenate dimeglumine on cell viability by exposing primary human chondrocytes to clinically relevant solutions (either 1.7 mmol/L, 2.5 mmol/L, 5 mmol/L or 10 mmol/L) for 1 hour, 4 hours, 16 hours or 24 hours. No evidence of cytotoxicity was found. At all time points, across all doses, gadolinium-contrast-treated cultures yielded similar formazan staining patterns as untreated media controls. This is in keeping with studies on the pharmacokinetics and pharmacodynamics of gadolinium chelates (13, 19, 31). Although Gd^{3+} is highly toxic, the free gadolinium concentration after intra-articular administration of gadolinium-contrast agents is probably very low. This has been demonstrated *in vitro* by Brown et al. (33) who mixed and incubated three iodinated contrast agents with gadopentetate dimeglumine. No significant dissociation of free toxic gadolinium ion was found from the gadopentetate dimeglumine complex, even after the addition of saline, lidocaine and epinephrine. The authors concluded that the mixture of iodinated and gadolinium-based contrast agents is safe and can be combined with lidocaine or epinephrine for intra-articular use. Additionally, intra-venous administration of Gd-contrast

agents up to concentrations of 0.3 mmol/L/kg body weight have been found to be safe in adults. Although no precise figures for the total body does exist in the case of direct arthrography, with a typical injection concentration of 1 to 2 mmol/L, the whole body dose of gadolinium following intra-articular administration is much lower than the maximum permissible and approved intravenous dose (20, 31) (Section 1.3.2).

Post-procedural pain is the main issue following direct arthrography (34, 35), yet the source of this pain remains unclear. Several reports have suggested that pain may be due to joint distension or a direct irritation of the synovium resulting in chemical synovitis (29, 35, 36). If pain is due to joint distension, then it would follow that most patients who undergo direct arthrography should develop post-arthrographic pain. However, Giaconi et al. (34) found that only 66% of their patients developed pain and reasoned that a patient-specific inflammatory response to chemical irritation was a more likely explanation. The effect of contrast agents on synovial tissues may be analogous to their apparent irritant effect on endothelium noted during venography (37). The latter is diminished by dilution of the contrast medium. The time course of pain after contrast agent injection may also be compatible with a diagnosis of chemical synovitis. Peak post-arthrographic pain has been reported to occur between 12 and 48 hours following injection of the shoulder before returning to baseline levels at 1 week (34, 38, 39). Several *in vivo* studies have demonstrated histological changes of the synovium up to 10 days after injection of contrast agents (19, 29, 40). In a rabbit model, Papacharalampous et al. (40) examined the effect of intra-articular contrast agents on knee synovial membrane at 1, 2, 10, 20, 30, 40 and 60 days after injection. They reported early, mild and transient histological changes and determined that it was the chemical structure (rather than the osmolality) of the contrast media which was the principal factor. The authors concluded that iodinated and gadolinium-based agents are well-tolerated for direct arthrography.

4.3 Chondrotoxicity of local anaesthetics

In our third set of experiments, we investigated the additive effect of local anaesthetic when mixed with either iohexol or gadopentetate dimeglumine in both a concentration-dependent and time-dependent fashion. Primary human chondrocytes were exposed to final concentrations of either iohexol 150 mg/ml, gadopentetate dimeglumine 2.5 mmol/L, lidocaine 0.5%, lidocaine 1% or a combination of iohexol + lidocaine (final concentrations of 150 mg/ml and 0.5%, respectively) or a combination of gadopentetate dimeglumine + lidocaine (final concentrations of 2.5 mmol/L and 0.5%, respectively). Cultures were exposed for 30 minutes, 45 minutes or 60 minutes and saline-treated cells served as the controls.

We found no evidence of toxicity following exposure to either iohexol or gadopentetate dimeglumine in isolation, with results of the cell viability assays equivalent to saline-treated controls at 30, 45 and 60 minutes. This supported our findings from our first and second sets of experiments. However, exposure of primary human chondrocytes to any concentration of lidocaine - either in isolation or in solution with iohexol or gadopentetate dimeglumine - led to a profound decrease in cell viability at each time point. Lidocaine exhibited dose-dependent cytotoxicity at 45 and 60 minutes on crystal violet staining and time-dependent effects were also evident. When compared to cell viability at 30 minutes, exposure to lidocaine 0.5% or lidocaine 1% led to further significant reductions at 45 and 60 minutes. Taken together, these observations imply an additive toxic effect of lidocaine when mixed with intra-articular contrast agents, which occurs in both a dose-dependent and time-dependent fashion.

Chondrocyte death is associated with cartilage degradation and osteoarthritis with a close correlation between the frequency of chondrocyte apoptosis and severity of osteoarthritic changes (41, 42). Because articular cartilage does not contain tissue macrophages, apoptotic and necrotic remnants cannot be removed and remain to cause further tissue damage (43). Therefore, any intra-articular agents with potential cytotoxicity should be used with caution. Studies have revealed that longer periods of exposure and higher concentrations of local anaesthetics are more toxic to both chondrocyte monolayers and cultured explants. These time- and dose-toxicity relationships have been demonstrated for lidocaine, bupivacaine, levobupivacaine, ropivacaine and mepivacaine (44, 45). Of the amide local anaesthetics, ropivacaine appears to be the least toxic at doses less than 0.75% and bupivacaine appears to

be the most toxic at 0.5% and higher (32, 46-52). Our results are consistent with the published clinical and laboratory data on the chondrotoxic effects of lidocaine (46-48, 53-55).

The exact mechanism of local anaesthetic chondrotoxicity has not been fully elucidated. Feinstein et al. (56) found that bupivacaine potentiates nitric oxide synthase-2 activity in rat glial cells and astrocytes. Nitric oxide synthase-2 is normally absent from cells, but its expression and activity is induced on immunostimulation, suggesting that bupivacaine is capable of exacerbating an ongoing inflammatory process through production of nitric oxide. However, this property of bupivacaine has not been tested in human articular chondrocytes. Chemical incompatibility between synovial fluid and local anaesthetics may play a role (46, 57) while other studies have focused on mitochondrial dysfunction with subsequent cell necrosis or apoptosis. Grishko et al. (47) demonstrated mitochondrial DNA damage and a reduction in ATP and mitochondrial protein levels in response to treatment with a variety of local anaesthetics at varying concentrations. They also offered the possibility that some of the toxic effects of local anaesthetics are related to blockade of voltage-gated ion channels. Johnson et al. (58) found that lidocaine induced both necrosis and apoptosis in neuronal cells, with the mechanism of cell death dependent on anaesthetic dose. Cells exposed to 37 mM lidocaine for 30 minutes underwent cell death by apoptosis, while those exposed to 185 mM lidocaine for 10 minutes underwent necrosis. Similar results were found by Dragoo et al. (46) and it appears likely that the toxicity of lidocaine is related to mitochondrial dysfunction, with the actual mechanism of cell death dependent on the dose of local anaesthetic.

Although the cytotoxicity of local anaesthetics in chondrocyte cultures is unchallenged, the degree to which they inflict damage *in vivo* continues to be debated. Single intra-articular injections of local anaesthetic have been used for many years without detrimental long-term clinical effects and many authors feel that the real threat lies with continuous infusion protocols (45, 59). The range of doses and time exposure of chondrocytes after a single intra-articular injection is reduced *in vivo* by a variety of factors, including joint fluid, bleeding and local anaesthetic absorption and clearance. The maximum blood concentration of bupivacaine has been observed clinically to occur between 35 to 40 minutes after a bolus injection into the knee (60). These findings indirectly show that systemic absorption alone substantially reduces the effective intra-articular concentration of bupivacaine within this time frame. In contrast, post-operative pain pumps overcome these dilutional effects and continuous high-dose exposure increases the amount of local anaesthetic that diffuses through the cartilage matrix. Integrity of the articular cartilage also appears to play a role and cartilage disruption

(e.g. by the insertion of suture anchors) has been clinically observed to increase the risk of chondrolysis in shoulders receiving post-operative pain pumps (45, 61). These findings have been confirmed in laboratory studies. Chondrocytes cultured in monolayers or in alginate beads are not protected by surrounding ECM and are therefore more susceptible to lower doses of local anaesthetic than chondrocytes embedded within intact cartilage (45, 62). When the superficial layer of cartilage is damaged, local anaesthetics can more easily reach the chondrocytes within the matrix. Chu et al. exposed bovine tissue explants to varying doses of bupivacaine and found that cell death rates were more pronounced in specimens which had the top 1 mm of cartilage removed (63, 64). However, an intact superficial layer is less able to protect chondrocytes from the toxic effects of lidocaine, which diffuses more easily through the matrix because of its smaller molecular weight (65). Moreover, local anaesthetics have been shown to cause substantial chondrocyte necrosis despite a normal histological appearance. In further work by Chu et al. (66), the effect of a single intra-articular injection of bupivacaine 0.5% was examined in a rat model. Subsurface pathological changes that were detected on fluorescent and confocal microscopy were not observed macroscopically, indicating that toxic effects of local anaesthetics may be not be evident on clinical inspection and could take several years to develop. Fewer chondrocytes to repair and maintain the extracellular matrix could potentially reduce future tolerance to mechanical loading and injury (67).

While animal studies permit the use of advanced laboratory techniques to evaluate the *in vivo* effects of intra-articular local anaesthetics, no animal model precisely replicates the thickness, structure, size, biomechanics and repair potential of human articular cartilage (62, 66). Questions remain, therefore, and despite the apparently uncomplicated use of intra-articular bolus doses of local anaesthetics for many years, interest in their potential toxicity continues.

4.4 Limitations

Although this study is perhaps limited by its *in vitro* nature - it is unknown how closely *in vitro* research mimics the *in vivo* state - monolayer culture of chondrocytes has been well established as a method of assessing *in vitro* cell response to various treatments (44, 59). Although we harvested cartilage from an older patient population (median age, 74 years) only intact specimens were used. Therefore, chondrocyte monolayers were prepared from human articular cartilage in which there was no evidence of osteoarthritis. By contrast, in the only other study to use human articular cartilage, Oznam et al. (28) harvested specimens from patients with established osteoarthritis at the time of total knee arthroplasty. Moreover, they did not verify chondrocyte phenotype in their work. Nonetheless, we acknowledge that intact articular cartilage may be partially protected from the cytotoxic effects of local anaesthetic (63, 68) and more complex models incorporating three-dimensional scaffolds may offer a more realistic representation of *in vivo* chondrocyte behaviour. These models may be the future gold standard for *in vitro* analysis and use of a common model would allow for better comparison between individual studies.

This study is also limited by the use of constant concentrations of contrast agent and/or local anaesthetic as a model for a single injection. The pharmacokinetics of small molecule drugs injected intra-articularly result in multi-exponential decay over the time course of hours (46, 69, 70) and the exact time that a drug is acting at a set concentration is unknown. In light of this, we employed a range of clinically relevant concentrations, across various time points, in a monolayer culture which is likely to estimate, or over-estimate, the cytotoxic effects of each agent. Therefore, the lack of chondrotoxicity of intra-articular contrast agents in this model is reassuring.

Finally, we only investigated one local anaesthetic agent. However, extensive work has already been completed on the chondrotoxicity of local anaesthetics and we intended to select the most commonly used local anaesthetic agent in direct arthrogram clinical solutions.

4.5 Conclusion

Our *in vitro* results reveal that gadolinium-based contrast agents are safe for intra-articular injection at the concentrations routinely used in clinical practice.

While prolonged exposure to higher doses of iodine-based contrast agents did result in a statistically significant decrease in cell viability in comparison to a media control, this may reflect nutrient deprivation rather than a true cytotoxic effect. Moreover, no evidence of time-dependent effects were observed across 1, 4, 16 and 24 hour exposure to either iodine-based agent at 50 mg/ml and 200 mg/ml.

Orthopaedic surgeons and musculoskeletal radiologists need to be aware, however, that the addition of lidocaine to direct arthrography solutions carries a dose-dependent and time-dependent cytotoxic effect in a monolayer culture of primary human chondrocytes. Based on our findings, it may not be advisable to add local anaesthetic to intra-articular contrast agents when preparing direct arthrography injection solutions.

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