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Simulated impact trauma and osteoarthritis: the role of cell volume and mechanotransduction

Yanitsa Nedyalkov Nedelcheva

School of Life Sciences

SIMULATED IMPACT TRAUMA AND OSTEOARTHRITIS - THE ROLE OF CELL VOLUME AND MECHANOTRANSDUCTION

YANITSA NEDYALKOVA
NEDELCHEVA

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degree in this or any other university.*

Abstract

Health and emotional benefits are linked to participation in exercise, however single-impact load (e.g. trauma) and altered joint loading can cause bone fracture(s) resulting in permanent cartilage damage and increased risk of osteoarthritis (OA).

This study investigated the role of single mechanical load and the effects of chondroprotective agents over short periods of time (2-30 min) post impact. Mechanical load (force 1.14 N) induced by a drop tower device caused cell death from as early as 2 min. Pre-incubation in hypertonic media protected chondrocytes from cell death, whereby at 30 min, death was decreased from 9.22 % to 3.42 % ($p<0.01$), thus implicating volume regulatory changes as a potential key mechanism for chondroprotection, with *in situ* chondrocytes altering their cell volume in response to hypertonicity by 20 %. Investigation of the cell cytoskeleton, showed that hypertonicity increased cortical actin by 29 % within the superficial zone (SZ) only ($p<0.01$).

Volume and actin polymerisation regulation are governed by intracellular Ca^{2+} and the regulatory volume decrease (RVD) inhibitor REV5901 has been linked to both and was therefore tested for its potential chondroprotective properties. Impact led to cell death, which was significantly reduced by REV5901 from 10.92 % to 5.44 % ($p<0.001$) and initial cell volume was reduced by 25%. Cortical actin staining was increased by 20 % within the SZ of articular cartilage only ($p<0.01$). $GdCl_3$ (blocker for stretch-activated calcium channels), uridine-5'-triphosphate (uridine; calcium mobilizing mediator) and the phosphoinositide 3-kinases (PI3K) inhibitor wortmannin were used to determine its mechanism of action. Wortmannin alone increased cell death and inhibited REV5901 chondroprotective effects with no alteration in cell volume compared to REV5901 alone. Whilst uridine reduced cell volume with 20 % ($p<0.05$) it did not reduce cell death significantly from 10.92 % to 8.10 % ($p>0.05$) compared to control. $GdCl_3$ inhibited REV5901 chondroprotective effects by increasing cell death by ~ 5 % compared to control ($p<0.05$), F-actin staining appeared reduced 72.84 AU and not significantly different from control ($p>0.05$).

The role of the cell cytoskeleton is important for cell mechanotransduction and for maintaining integrity as actin microfilaments are recognized to bear tension therefore, alterations of the actin-binding proteins responsible for actin treadmilling cofilin, profilin and gelsolin mRNA was compared to untreated chondrocytes. REV5901 was observed to reduce cofilin (known actin depolymerizing factor) with 30 % ($p<0.05$) and increase profilin significantly by 75 % ($p<0.001$) respectively. Western blot analysis showed that only cofilin and gelsolin were expressed in all samples with no detection of profilin. REV5901 was observed to significantly reduce cofilin by 28 % ($p<0.05$).

This data highlights that REV5901 exhibits chondroprotective properties in part due to the polymerisation of the actin cytoskeleton *via* PI3Ks pathway. This could offer a novel therapeutic opportunity for perention of irreversible cartilagedamage following acute impact trauma.

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Abbreviations

$[Ca^{2+}]_0$:	extracellular calcium
$[Ca^{2+}]_i$:	intracellular calcium
2D:	Two-Dimensional
3D:	Three-Dimensional
5-LO:	5-Lypoxygenase
ADAMTS:	A Disintegrin And Metalloproteinase with Thrombospondin Motifs
ATP:	Adenosine Triphosphate
AVD:	Apoptotic Volume Decrease
Bak:	Bcl-2 homologous antagonist/killer
Bax:	Bcl-2-associated X protein
Bcl-2:	B-Cell Lymphoma 2
BPS:	Basic Physiological Saline
BSA:	Bovine Serum Albumin
Ca^{2+}:	Calcium
$CaCl_2$:	Calcium Chloride
CLSM:	Confocal Laser Scanning Microscope
Col2:	Collagen Type II
Cyt c:	Cytochrome C
ddH_2O:	Double Distilled Water
DISC:	Death-Inducing Signaling Complex
DMEM:	Dulbecco's Modified Eagle's Medium
DMSO:	Dimethyl Sulfoxide
DZ:	Deep Zone
ECM:	Extracellular Matrix
EGTA:	Ethylene-Glycol-Tetraacetic Acid
F-actin:	Filamentous Actin
FADD:	Fas Associated Death Domain
G-actin:	Globular Actin
Gd^{3+}:	Gadolinium
$GdCl_3$:	Gadolinium Trichloride
IL:	Interleukin
IP_3:	Inositol Triphosphate

LTB$_4$:	Leukotriene B$_4$
MMP:	Matrix Metalloproteinase
mOsm:	Miliosmole/Kg H$_2$O
MZ:	Mid Zone
NaCl:	Sodium Chloride
NCX:	Sodium Calcium Exchanger
NH$_4$Cl:	Ammonium Chloride
NO:	Nitric Oxide
OA:	Osteoarthritis
PBS:	Phosphate Buffer Saline
PDC:	Percent Dead Cellc
PFA:	Paraformaldehyde
PG:	Proteoglycan
PGE$_2$:	Prostaglandin E$_2$
PI:	Propidium Iodide
PKC:	Protein Kinase C
PLCβ_3:	Phospholipase C β_3
REV 5901:	α- pentyl- 3- (2- quinolinylmethoxy)-benzene methanol
RFP:	Red Fluorescent Protein
ROS:	Reactive Oxygen Species
RVD:	Regulatory Volume Decrease
RVI:	Regulatory Volume Increase
SACC:	Stretch Activated Calcium Channel
SZ:	Superficial Zone
TNF-α:	Tumor Necrosis Factor α
TNFR:	Tumor Necrosis Factor Receptor
TRITC:	Tetramethyl Rhodamine Iso-Thiocyanate
TUNEL:	Terminal deoxynucleotidyl transferase dUTP Nick End Labeling
UK:	United Kingdom of Great Britain and Northern Ireland
VSOAC:	Volume Sensitive Organic Anion Channel
Wortmannin	WMN

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CHAPTER 1

1. INTRODUCTION

Numerous and essential health and emotional benefits are linked to the participation in exercise (Bauman, 2004, Buckwalter and Martin, 2004, Peluso *et al.,* 2005, Wang *et al.,* 2011, Hamer *et al.,* 2012) but in high-impact, load-bearing sports (e.g. football, tennis, running, boxing) mechanically elicited cartilage trauma is a major concern as many of them are linked to trauma and concomitant risk of joint failure in later life (Saxon *et al.,* 1999, Lequesene, 2004, Ross, 2005). Indeed, whilst retrospective cohort studies support the conclusion that moderate exercise does not increase the predisposition to osteoarthritis (OA), there is evidence that high impact sport increases the risk of OA (Spector *et al.,* 1996, Urquhart *et al.,* 2007). Sports injury that cause joint instability or cartilage trauma is a major concern in high-impact, load-bearing sport and directly linked to the onset of post traumatic OA (Saxon *et al.,* 1999, Lotz and Kraus, 2010, Stehling *et al.,* 2012). Therefore, the elucidation of cartilage damage as a consequence of mechanical trauma is of paramount importance for both the casual, professional and elite sports person.

1.1   Cartilage and Chondrocyte

1.1.1 Cartilage and its structure

Articular cartilage is semi-opaque, grey or white, dense and highly specialised connective tissue capable of withstanding considerable pressure and can be found on the opposing surface of the synovial joints (Stockwell, 1991, Buckwalter, 2002). Lacking both a blood and nervous supply it is an avascular, aneuronal and alymphatic tissue (Wilkins *et al.,* 2000, Goldring *et al.,* 2011) and in conjunction with synovial fluid, it provides a smooth, low friction surface suitable for joint articulation therefore protecting the underlying bone from shearing forces generated by load (Matthews *et al.,* 1986, Darling *et al.,* 2006). Three types of cartilage exist: fibro, hyaline and elastic cartilage whereby hyaline (also called articular cartilage), is the type of cartilage that lines the articulating surfaces of long bones in diarthrodial joints (Ratcliffe and Mow, 1996, Nordin and Frankel, 2001). Thin cartilage is found in congruent joints such as the ankle, whereas thicker cartilage is found in

incongruent joints such as the knee (Muir *et al.*, 1970, Henden and Beeson, 2009).

Articular cartilage is comprised of an extracellular matrix (ECM) synthesised by the chondrocytes which represent ~5 % of the tissue volume (Stockwell, 1987). Chondrocytes are the sole resident cell type in articular cartilage (Stockwell, 1979, Goldring and Goldring, 2007) and thus play a crucial role in the organisation, maintenance, synthesis and turnover of extracellular matrix (ECM) (Chahine *et al.,* 2013). Chondrocytes produce and maintain the ECM in response to the changing environmental pressures that is modulated by joint loading with enhanced loading stimulating matrix synthesis (Urban, 1994, Wilkins, 2003, Goldring *et al.,* 2011) and in return they are protected from potential damaging forces of mechanical loads by the very same ECM they produce (Howell, 1986, Hall, 1998, Guilak *et al.,* 2001, Goldring 2012b).

The ECM is a macromolecular construction that plays an extensive role in variety of different functions and is comprised of a cross network consisting of collagen fibrils and proteoglycans (PGs), specifically hyaluronan and the highly negatively charged molecule aggrecan. In addition, there are a small number of non-collagenous glycoproteins that contribute to tissue regulation (Durr *et al.,* 1996, Poole, 1997). Collagen is a major protein of ECM and there are various different types of collagens: type I, II, III, VI, X, or XI, however, Collagen II (col2) is the most abundant primary collagen of articular cartilage, comprising of 80-90 % of the total collagen content in healthy adult articular tissue (Bacerra *et al.,* 2010), forming a dense fibrous network which is responsible for supporting the cells against shear stress (Guilak, 1995, Muir, 1995, Eyre *et al.,* 2006, Goldring and Goldring, 2007, Goldring and Macu, 2009). It forms heteropolymer complexes with other cartilage collagens such as collagen IX and XI (Goldring, 2012b). Collagen Type VI is present in different cartilage types and it has a distinctive filamentous network that is concentrated around the chondrocyte and therefore this protein is a hallmark for chondrons microanatomy (Figure 1.1), whilst collagen X is restricted to transition areas such as the calcified zone and is distinguished by its short non-fibril-forming structure that surrounds cells as

in a hypertrophic zone (Poole, 1997, Nordin and Frankel, 2001, Eyre *et al.,* 2006, Goldring, 2012b).

Proteoglycans (PGs) are negatively charged molecules that comprise of long repeated linear chains of specific disaccharides called glycosaminoglycans (GAG) attached to a core protein. Aggrecan, one of the most common and predominant PG in cartilage (Milner, Willkins and Gibson, 2012) binds Hyaluronic acid (HA) and GAG short side chains: chondroitin sulphate (CS) and keraftan sulphate (KS) (Goldring, 2012b). PGs are responsible for providing articular cartilage with the ability to resist tension (Urban, 1994), and due to their negative change they attract water and cations becoming hydrated, resulting in an expansion of up to five times their original size (Stockwell, 1991, Wilkins *et al.,* 2000). This causes the PG to swell and become trapped in the collagen meshwork during joint loading, during this process hydrostatic pressure is raised and ECM deforms. When load is removed, PGs re-embed water and achieve a new equilibrium, thus allowing cartilage to maintain a steady state (Hall *et al.*, 1996). The 'trapped' nature of the PGs within the dense collagen network shapes an extraordinary ionic environment that has an uneven distribution of charged ions as a result of impermeable or immobilised charged molecules and is described by the Gibbs-Donnan equilibrium (Maroudas, 1979).

Articular cartilage also incorporates several other proteins responsible for the regulation and turnover of the ECM, anchorin and fibronectin (Goldring and Macu, 2009). Fibronectin, is a dimeric glycoprotein that regulates cell adhesion and differentiation (Glant *et al.,* 1985), whiles anchrorin is a collagen binding protein (Hofmann *et al.,* 1992).
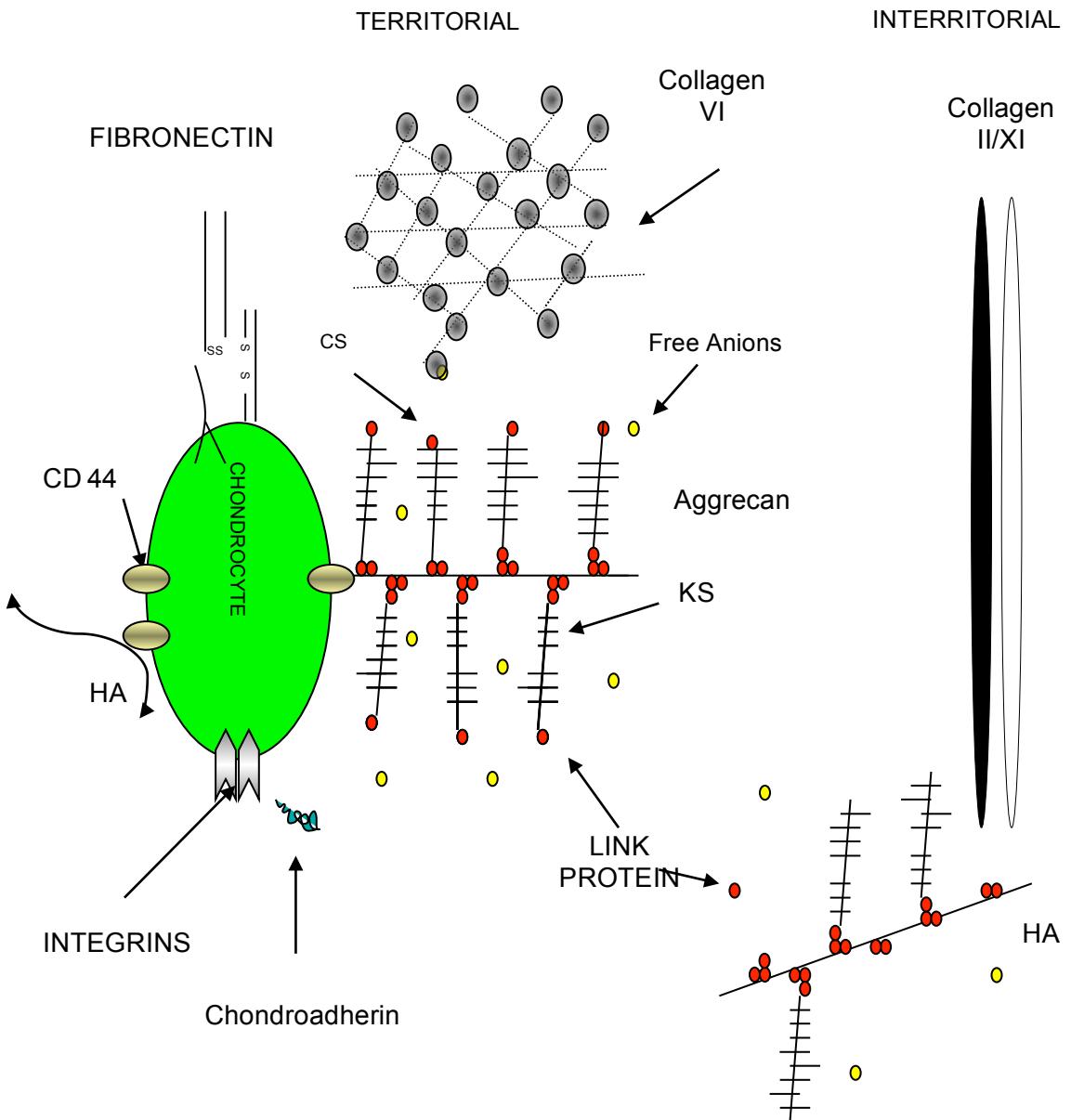
Figure 1.1 Components in the cartilage extracellular matrix.

Molecular organisation of territorial and interritorial matrix (Diagram is not drawn to scale). Illustration of cartilage ECM presenting the different molecular organization of close to cell (territorial) and distant from cell (interritorial) space. Within the territorial area major constituents are collagen II and VI and proteoglycans (aggrecan, hyaluronic acid (HA), keraftan sulphate (KS) and chondroitin sulphate (CS)). CD44 is a cell surface receptor for HA.

1.1.2 The Chondrocyte

This terminally differentiated cell (Goldring, 2000) is the unique cell type within articular cartilage and in the human femoral head, they reside at a density of ~10,000 chondrocytes/μm^3 (Stockwell, 1979, Goldring, 2012a). Chondrocytes operate at low oxygen levels and are entirely reliant on nutrients being provided by diffusion from the articular surface due to the avascular nature of the tissue.

Articular cartilage is arranged into four distinct zones, superficial/tangential zone (SZ) situated at the top of the cartilage layer, followed by the mid zone (MZ), characterized with the highest content of GAG, deep/radial zone (DZ), and the calcified zone (Stockwell, 1991, Figure 1.2). The protective properties of the cartilage are a consequence of the distinct zones and each zone is characterised by the content of collagen, PGs and the shape/size of chondrocytes within it. Organization of collagen within the SZ represents 10-20 % of the cartilage thickness (Kim *et al.,* 1994) and consists mainly of collagen type II, which is oriented in parallel to the articular surface (Weiss *et al.,* 1968). Chondrocytes within this zone are elongated and situated in the shear stress plane of the articular surface at an approximate volume 454 ± 18 μm^3 (Urban, 1994, Bush and Hall, 2003). Conversely, within the MZ, collagen fibrils are randomly situated in pairs, and the chondrocytes appear round in shape and often arranged in pairs. MZ represents about 40-60 % of cartilage thickness and is often referred to as transitional to the DZ, with a cell volume estimated 553 ± 15 μm^3 (Urban, 1994, Bush and Hall, 2003). Within the DZ ellipsoidal shaped chondrocytes are arranged in groups (stacks) where cells are situated in the middle with collagen fibres distributed between radially oriented collagen fibres. This crosses the tidemark and fastens the cartilage to the bone at the calcified zone, this distinct tidemark is considered as a boundary between DZ and the bone, thus separating the non-calcified tissue from the calcified tissue, with a volume of 805 ± 79 μm^3 (Guilak, 1995, Bush and Hall, 2003, Athanasiou *et al.* 2004, Darling *et al.,* 2004).

Superficial Zone

Mid Zone

Deep Zone

Tide Mark

Calcified Zone

200μM
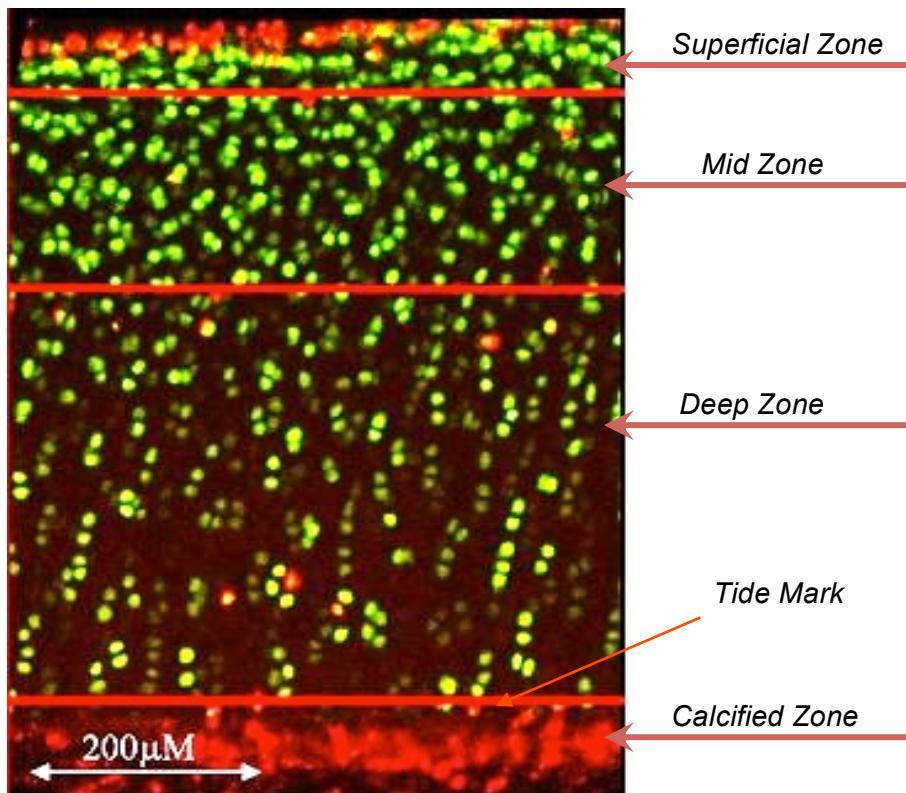
Figure 1.2 Representative Distribution of zones into cartilage.

Full depth bovine articular cartilage explants were removed aseptically from metacarpal phalangeal joint and incubated with calcein-AM (green) and propidium iodide (red). Image was acquired by confocal laser scanning technique and clearly shows the three distinctive zones and the calcified zone. SZ cells are elongated and aligned in parallel, MZ cells are rounded and situated in pairs and DZ chondrocytes in clusters of elongated cells. At the very end, is situated the calcified zone separated from the other three zones by a tidemark. Image shows the coronal CLSM projection of cartilage, acquired by Dr P. G. Bush, adapted with permission.

1.2 Cytoskeletal Organisation

The mechanical response of the cell to loading is dependant on mechanical properties, cell morphology and ECM interactions all of which are influenced by the cytoskeleton (Chahine *et al.,* 2013). The cytoskeleton comprises of a dynamic 3D network including actin microfilaments, tubulin microfilaments and vimentin intermediate filaments (Benjamin *et al.,* 1994, Henson and Vincent, 2008, Blain, 2009). The organization of these cytoskeletal networks have been described in full-depth explants, isolated primary cells and monolyer (Durrant *et al.,* 1999, Trickery *et al.,* 2004, Sasazki *et al.,* 2008). Within bovine articular cartilage, F-actin is distributed uniformly throughout the tissue depth, conversely to both vimentin and tubulin, which are predominantly detected within the SZ compared to DZ chondrocytes (Langelier *et al.,* 2000). In monolayer culture, chondrocytes have an ameboid shape and their diameter is greater than both *in situ* and 3D cultured chondrocytes, whilst the cytoskeleton is predominately located just beneath the cell membrane (Sasazki *et al.,* 2008).

Tubulin microtubules have the ability to self assemble from the polymeric microtubular structure and exist as dimeric proteins with an α and β structure, where by each monomer has a molecular mass of 55 kDa (Valiron *et al.,* 2009). Within chondrocytes there is a loose distribution of these proteins and they are not thought to be essential for cell integrity (Langelier *et al.,* 2000, Trickery *et al.,* 2004), whilst mechanical loading has been shown to have no effect on tubulin concentration and distribution.

Vimentin intermediate filament networks within chondrocytes are highly dynamic in nature, whereby all filaments have similar structural features. These highly organised proteins spread within the cytoplasm and connect the nucleus with the cell periphery (Trickery *et al.,* 2004). By examining the cytoskeletal architecture of chondrocytes in 3D cultures, it was recently reported that vimentin filaments represent a less dynamic and more rigid structure relative to the actin filaments (Steklov *et al.,* 2009, Haudenschild, 2011). Vimentin filaments are the most prominent filament within weight

bearing regions and static load increases their organisation (Eggli *et al.,* 1988, Henson and Vincent, 2008). In conjunction with actin filaments, the vimentin network plays a crucial role in the chondrocytes integrity and mechanotransduction abilities (Trickery *et al.,* 2004, Blein *et al.,* 2006, Henson and Vincent, 2008, Steklov *et al.,* 2009).

Actin, a globular protein (termed G-actin) of 43 kDa consists of three different monomers (α, β and γ; Blain, 2009) and has been reported to be the most abundant protein in eukaryotic cells (Disanza *et al.,* 2005). Under physiological conditions G-actin forms highly organised polymer filaments called filamentous actin (F-actin; Figure 1.3). This is formed by polymerisation of G-actin that has been shown to be Mg^{2+}/Ca^{2+} and ATP dependant. This ATP-dependant process of actin filament formation is reversible, and when ADP-actin monomers undergo nucleotide exchange producing ATP–actin they undergo a new round of polymerisation in a process of assembly and disassembly termed 'treadmilling' (Pollared and Borisy, 2003). The 'treadmilling' of actin filaments is regulated by variety of actin binding proteins that promote nucleation of actin such as profilin and Arp2/3 complex (Goley and Welch, 2006), or filament depolymerisation such as actin-depolymerising factor (ADF) and cofilin (Ono, 2007, Bamburg and Bernstein, 2010), and those that cap the filament ends such as gelsolin (Cooper and Schafer 2000, Erickson *et al.,* 2003).

The organisation of F-actin has been implicated to be involved in cartilage chondrocyte mechanics and mechanotransduction (Guilak 1995, Grodzinsky *et al.,* 2000, Erickson *et al.,* 2003, Darling *et al.,* 2006, Campbell *et al.,* 2007) and is influenced by mechanical loading, osmotic environment and inflammation, all of which are associated with OA (Table 1.1; Li *et al.,* 2007). Manipulation of external osmotic pressure results in remodelling of the actin cytoskeleton, whereby hypo-osmotic stress leads to F-actin depolymerisation and reorganisation (Chao *et al.,* 2006, Blain, 2009). Mechanical pressure can alter F-actin in a load dependent fashion (Knight *et al.,* 2006) whereby increasing pressure to 15 MPa leads to a decrease in F-actin which

completely depolymerises when the pressure increases to 30 MPa (Parkkinen *et al.,* 1995). However, actin-binding proteins such as ADF and cofilin are significantly increased after mechanical stimulation of chondrocytes and occurred together with temporal disassembly of F-actin (Campbell *et al.,* 2007).

Chondrocyte	F-actin organisation Characteristics	Reference
Healthy	Cortical distribution	*Blain et al., 2006, Sasazki et al., 2008*
Mechanical Loading	Load dependent, reversible loss of actin fibres	*Blain et al., 2006, Campbell et al., 2007, Knight et al., 2006, Parkkinen et al., 1995, Trickery et al., 2004,*
Osmotic Stress	Hypo-osmotic: prompts decreased actin fibres (depolymerised) Hyperosmotic: polymerised, with well defined fibres	*Blain, 2009, Chao et al., 2006, Erickson et al., 2003*
OA	Less defined, disassembled and localised in the cytoplasm	*Li et al., 2007*

Table 1.1 Organisation of the F-actin in healthy, osteoarthritic and stressed articular chondrocytes.

The organisation of F-actin in healthy chondrocytes has been described to be cortically and peripherally localised. Mechanical loading alters actin in a load dependent fashion where by manipulation of external osmotic pressure results in remodelling of the actin cytoskeleton. Distinguishing criteria are characterised here. (Adapted from Blain, 2009).
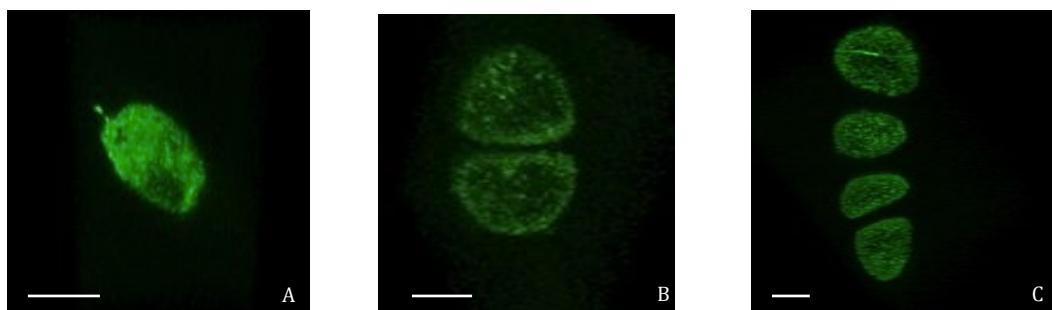
Figure 1.3 The F-actin organisation of *in situ* bovine chondrocytes.

Bovine articular cartilage explants were isolated from metacarpo-phalangeal joints and fixed in 4 % Para-formaldehyde, and labelled with Alexa-488 Phalloidin. Explants were visualized via CLSM. Chondrocytes from the three distance zones (A) SZ, (B) MZ, (C) DZ were presented. Actin was predominantly localised to the periphery of the cell. Bar = 5 µm

1.3 Mechanotransduction in articular cartilage

The main functions of articular cartilage are concerned with load-bearing (Urban, 1994) and articular chondrocytes are responsible for the mechanical properties of cartilage and its biochemical composition. They are the only resident cell type within the tissue and are responsible for matrix synthesis and turnover. During normal activities, loading pressure in cartilage may rise to 10-20 MPa within milliseconds (Milner, Wilkins and Gibson, 2012). Whilst this mechanical loading is essential for the synthesis of a viable ECM (Wilkins, 2000) excessive mechanical loading leads to cartilage damage and the formation of tissue lesions or fissures, that comprise the integrity of tissue and increase the risk of development of secondary OA (Chen *et al.,* 2005, Griffin and Guilak, 2005, Verteramo and Seedhom, 2007, Bader *et al.,* 2011, Goldring, 2012).

Abnormal joint loading or simulated excessive mechanical loading utilising a drop-tower (where by a mass is dropped onto cartilage from a defined height), can result in imbalance in cartilage metabolism and can mimic the impact force experienced during high-impact trauma (Jeffrey *et al.,* 1995, Aspden, 2002, Jeffrey and Aspden, 2006). High-impact mechanical loading leads to damage to the collagen network, resulting in tissue swelling and chondrocyte death through apoptosis or necrosis (Borrelli *et al.,* 2003, Huser and Davies, 2006, Yeow *et al.,* 2009, Han *et al.,* 2011,). However, a complete understanding of the events involved in chondrocyte mechanotransduction is still unknown (Han *et al.,* 2011, Mobasheri *et al.,* 2012). A number of stimuli including compression and shear stress are associated with alterations in the cell shape, with the actin cytoskeleton able to influence the capacity for volume regulation (Kerrigan and Hall, 2007, Raizman *et al.,* 2010, Lewis *et al.,* 2011). This process is linked to volume-regulatory conditions including regulatory volume increase (RVI) and regulatory volume decrease (RVD; Jeffrey, 1995, Wilkins, 2000, Kerrigan *et al.,* 2006, Lewis *et al.,* 2011). During walking, the pressure on cartilage oscillates between 0.2 MPa and 0.5 MPa (Broom and Myers, 1980) thus

resulting in deformation of chondrocytes, leading to an increase in extracellular osmolarity, an effect that is reversible in healthy cartilage upon removal of the load (Maroudas and Bannon, 1981). Abnormal static compression (above 0.5 MPa) causes irreversible damage to cartilage and chondrocyte metabolic activities are impaired (Burton-Wurster *et al.*, 1993, Guilak *et al.*, 1994). Importantly pressure induces a set of cellular responses including calcium rise (Valhmu *et al.*, 1998), up-regulation of aggrecan and collagen type II, and increased production of matrix metalloproteinase 3 (MMP-3) (Lee *et al.*, 1999, Sadler *et al.*, 2000).

1.3.1 Mechanotransduction and Volume Regulation

The mechanisms by which chondrocytes alter their biological activity in response to joint loading are not fully understood but various biochemical and bio-physiological pathways are involved (Guilak *et al.,* 2001, Mobasheri *et al.*, 2012, Qusous *et al.,* 2012). Normal physiological loading exposes chondrocytes to mechanical and osmotic stress, fluid pressure and fluid flow and in response to these stimuli. Water is exuded from the cartilage tissue and chondrocytes activate their volume regulatory mechanisms including RVD and RVI (Guilak, 1995, Kerrigan *et al.,* 2006). Cell volume regulation is important to chondrocyte physiology and the mechanisms involved include the activation of membrane transporters that help cells to mediate dynamic volume equilibrium necessary for optimal physiological and metabolic process (Raizman *et al.,* 2010, Lewis *et al.,* 2011, Mobasheri *et al.*, 2012).

Freshly isolated and *in situ* chondrocytes have been reported to have limited response to RVI following hyperosmotic challenge compared to 2D cultured chondrocytes, suggesting that mechanotransduction mechanisms adapt with cell phenotype change (Kerrigan *et al.,* 2007, Lewis, *et al.,* 2011). The lack of RVI in *in situ* chondrocytes is not a phenomenon exclusive to this cell type as other cell types (e.g. lymphoid cells) (Hoffman *et al.,* 2009) have been reported to express similar features. However this effect on RVI varies in its response to swelling between the cells from the different chondrocyte zones (Bush and Hall, 2003).

Since RVD and RVI mechanisms are poorly understood, it has been suggested that the disruption of RVD mechanism may be linked to the onset for OA, as decreased osmolarity induces cell swelling (Hall, 1995, Wilkins *et al.,* 2000). Chondrocytes respond to volume decrease in a number of ways with activation of the Na^+-K^+-$2Cl^-$ co-transporter or other membrane co-transporters (Hall, 1995). Intracellular calcium ion concentration plays a role in regulatory volume control (McCarty and O'Neil, 1992, Han *et al.,* 2012) and it has been reported to increase in response to hyper-osmotic stress (Dascalu *et al.,* 1995, Lang *et al.,* 1998). In addition, Ca^{2+} is known to play a role in regulation of the Na^+/H^+ transporter and Ca^{2+}/calmodulin complex thus triggering the RVI (Pedersen *et al.,* 1996, Marchenko and Sage, 2000, Ong *et al.,* 2010).

1.3.2 Role of Calcium signalling in Mechanotransduction

The role of calcium signalling in chondrocyte mechanotransduction has been studied intensively over the past few years and the role of this ubiquitous intracellular secondary messenger is diverse and dependent on numerous factors including: (1) principal zone, (2) morphology, (3) culture conditions and (4) phenotype (Han *et al.,* 2012, Wann *et al.,* 2012). $[Ca^{2+}]_i$ has been linked to osmotic changes (Yelloweley *et al.,* 1999, Kerrigan *et al.,* 2006, Han *et al.,* 2012), fluid flow (Yelloweley *et al.,* 1999), pressure (Guilak *et al.,* 1999a) and membrane deformation (Guilak *et al.,* 1999b). With osmotic changes resulting in an increase in $[Ca^{2+}]_i$ shown to be mediated *via* the stretch-activated cation channel (SACC) induced by hypo-osmotic pressure (Kerrigan and Hall, 2008) and this response was inhibited by gadolinium chloride ($GdCl_3$, a specific SACC inhibitor). Similar observations of induced increase in influx of Ca^{2+} into the cell via SACC and release of Ca^{2+} in the intracellular store were reported in relation to fluid flow (Yellowley *et al.,* 1997, Yellowley *et al.,* 1999). Membrane deformation, as a result of fluid flow has been shown to induce an intracellular store - independent of a $[Ca^{2+}]_i$ rise which was inhibited by pharmacological inhibitors (e.g. taurine, tamoxifen, calmodulin) of sodium calcium exchanger (NCX) and SACC (Guilak, 1999).

1.4 Osteoarthritis

Osteoarthritis (OA) is the most common form of arthritis affecting millions of individuals worldwide (Goldring and Goldring, 2007, Loeser, 2011, Teeple *et al.,* 2013). The total cost of osteoarthritis to UK economy calculated by National Institute for Health and Clinical Excellence (NICE) is estimated to be about 1 % of the annual gross national product (NICE) with a total cost estimated around £5.7 billion (ARC, 2008).

OA is clinically referred to as a syndrome whose progression ultimately results in loss of cartilage function (Stockwell, 1991) and is associated with changes in the entire joint including the synovial joint lining, the surrounding connective tissue and cartilage (Loser, 2009). While the etiopathogensis of OA is not fully understood, historically the disease has been viewed as "wear and tear" disease, however studies suggest that OA involves a complex interplay of biochemical, mechanical and genetic factors (Pritchard and Guilak, 2006, Teeple *et al.,* 2013). Within the tissue numerous morphological and molecular changes occur including increases in cartilage hydration, loss of PGs, surface disruption, changes in chondrocyte morphology (Bush *et al.,* 2003, Han *et al.,* 2012), decrease in cell viability (Stockwell, 1991, Bush *et al.,* 2005, Goldring and Marcu, 2009), changes in collagen synthesis (Goldring, 2007), and production of pro-inflammatory cytokines including IL-1β, IL-6 and IL-8 as well as matrix-degrading enzymes (Fernandes *et al.,* 2002, Hedbom and Hauselmann, 2002, Goldring and Marcu, 2009, Natoli and Athanasiou, 2009, Goldring and Otero, 2011).

In the first stage, of the pathology, OA is characterised with changes of the cartilage surface which is no longer smooth (McDevitt *et al.,* 1977), as the disease progresses destruction of the collagen framework with subsequent loss of proteoglycans resulting in tissue hydration and elevations in chondrocyte volume leading to increased stiffness of the cartilage (Figure 1.4). Cells from the SZ usually flat become round and disappear from the tissue (Goldring and Marcu, 2009, Lonrenz and Richter, 2006). During the second stage, chondrocytes respond to the altered conditions of the ECM

and proliferate in an attempt to repair the tissue (Newman, 1998, Lonrenz and Richter, 2006). In the final stage, there is a decreased response in chondrocyte repair mechanisms, potentially due to the mechanical damage to the chondrocytes resulting in complete loss of cartilage tissue as a consequence (Lonrenz and Richter, 2006). The cartilage surface is broken by fissures and cracks can reach down to the calcified zone, loss of PGs is evident and reaches the DZ and the tide mark zone becomes unclear (Lorenz *et al.,* 2005, Goldring 2012a). At the cellular level, the presence of cytokines (IL-1β, TNFα, IL-6) and inflammatory mediators such as nitric oxide (NO), as well as matrix metalloproteinases (MMPs) lead to the synthesis and degradation of ECM (Tetlow *et al.*, 2001, Fernandes *et al.,* 2002, Bortner, 2004, Goldring, 2012b).
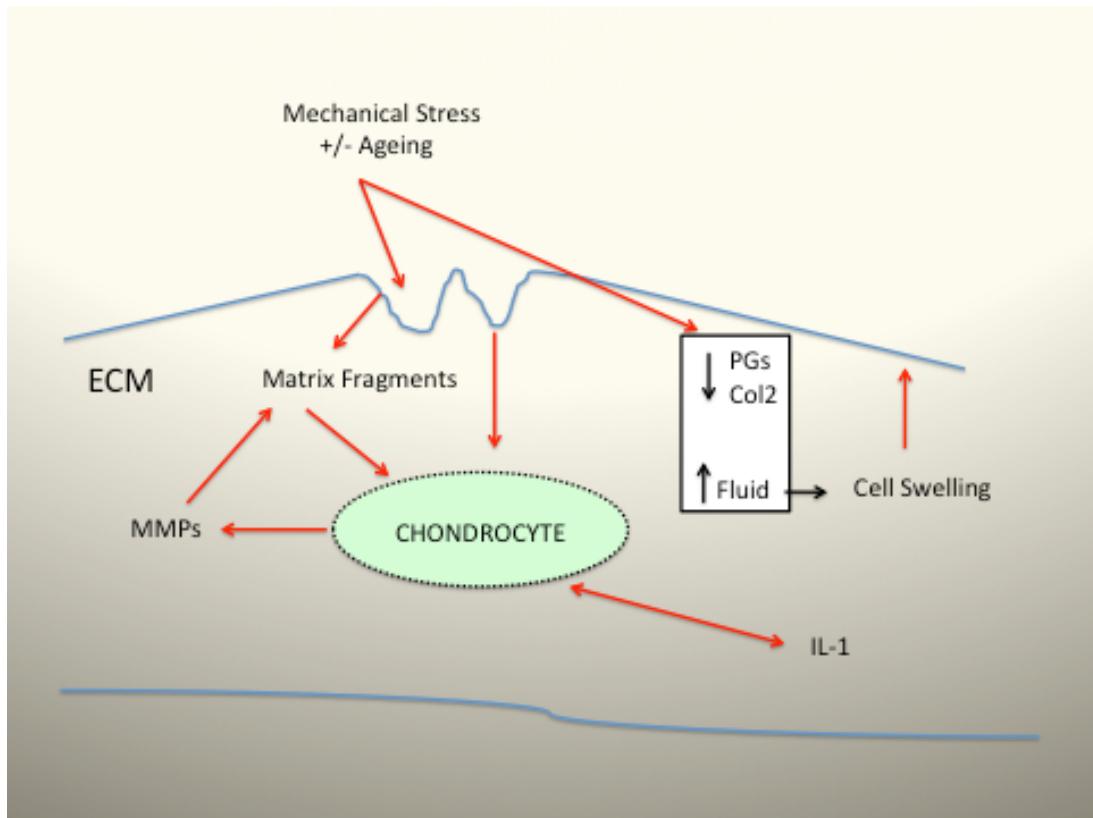
Figure 1.4 Model of the role of mechanical stress and biological factors in the pathogenesis OA.

Mechanical loading and biological factors such as aging result in tissue damage that induces increase of Collagen II (col2), proteoglycans (PGs) and matrix metalloproteinases (MMPs) production. Due to loss of the PGs the increased level of fluid causes cell swelling and the matrix fragmentation induces production of MMPs that result in further degradation of the extracellular matrix (ECM). To respond to the environmental changes, the chondrocytes release IL-1 that increases the effects on the matrix degradation and stimulates further production of IL-1. (Adapted from Goldring and Marcu, 2009).

1.4.1 Cell Death, Mechanical Load and Osteoarthritis

The pathogenesis of OA is still unclear and while mechanical load is an important regulator of cell function it can induce inflammatory mediators (IL-1β, NO) and MMP's (Fermor, 2001, Lotz, 2004), hence attempts have been made to distinguish between the type of death in response to mechanical load and inflammatory mediators. Apoptosis and necrosis are the two types of death that have been contrasted mechanically (Table 1.2). Apoptosis is programmed cell death, which could be physiological (cell renewal) or pathological (Kühn *et al.,* 2004, Kruz *et al.,* 2005). During the process of apoptosis cells loose their volume – termed apoptotic volume decrease (AVD; Aigner and Kim, 2002, Bortner, 2004) followed by condensation of cytoplasm and nucleosomal cleavage. This leads to mechanisms that results in controlled breakdown of the cell into apoptotic bodies, which are subsequently engulfed by phagocytes (Duprez *et al.,* 2009).

This cellular 'suicide' mechanism in mammalian cells can be activated by the intrinsic or extrinsic pathway (Duprez *et al.,* 2009) with various stimuli (e.g. cytotoxic insults or DNA fragmentation) triggering the intrinsic pathway leading to Bcl-2 protein family activation, whereby anti-apoptotic Bcl-2 proteins (Bax) inhibit pro-apoptosis. Once Bcl-2 proteins are activated *via* the intrinsic factors Bax, Bak inhibition is removed thus leading to formation of channels *via* which cytochrome c (cyt c) is released in the cytosol and associates with Apaf-1 and ATP, thus activating the enzymes crucial for the apoptotic cell death the 'executioner Caspases' (Caspase-3,-6 and-7) (Riedl and Salvesen, 2007, Youle and Strasser, 2008).

Stimulation of the extrinsic pathway leads to activation of 'death receptors' that belong to TNFR family (Fas, TNFR and Trail-R). Apoptosis is induced *via* the formation of death inducing signalling complex (DISC) that includes Fas associated death domain (FADD), which activates Caspase-8 and/or -10 and in turn activates the 'executioner Caspases' (Figure 1.5; Duprez *et al.,* 2009).

Type of cell death	Distinguishing Features	
	Morphologic Features	Physiologic Characteristics
Apoptosis	• Shrinking of cytoplasm and chromatin condensation • Nuclear fragmentation • Formation of apoptotic bodies • Affects individual cells	• Tightly regulated signalling events • Energy dependent • Enzymatically catalysed changes of cell membrane • Orderly fragmentation of DNA • Activation of caspases • Late loss of membrane integrity
Necrosis	• Swelling of cytoplasm and mitochondria • Total cellular disintegration • Affects groups of cells	• Early loss of membrane integrity • No energy requirement • Random destruction of DNA • DNA degradation occurs after membrane permeabilization

Table 1.2 Distinguishing features of apoptosis and necrosis.

Apoptosis and necrosis comprise a partially overlapping spectrum of characteristics. Criteria that distinguish apoptosis and necrosis are summarised here. (Table adapted from Kühn et al., 2004).
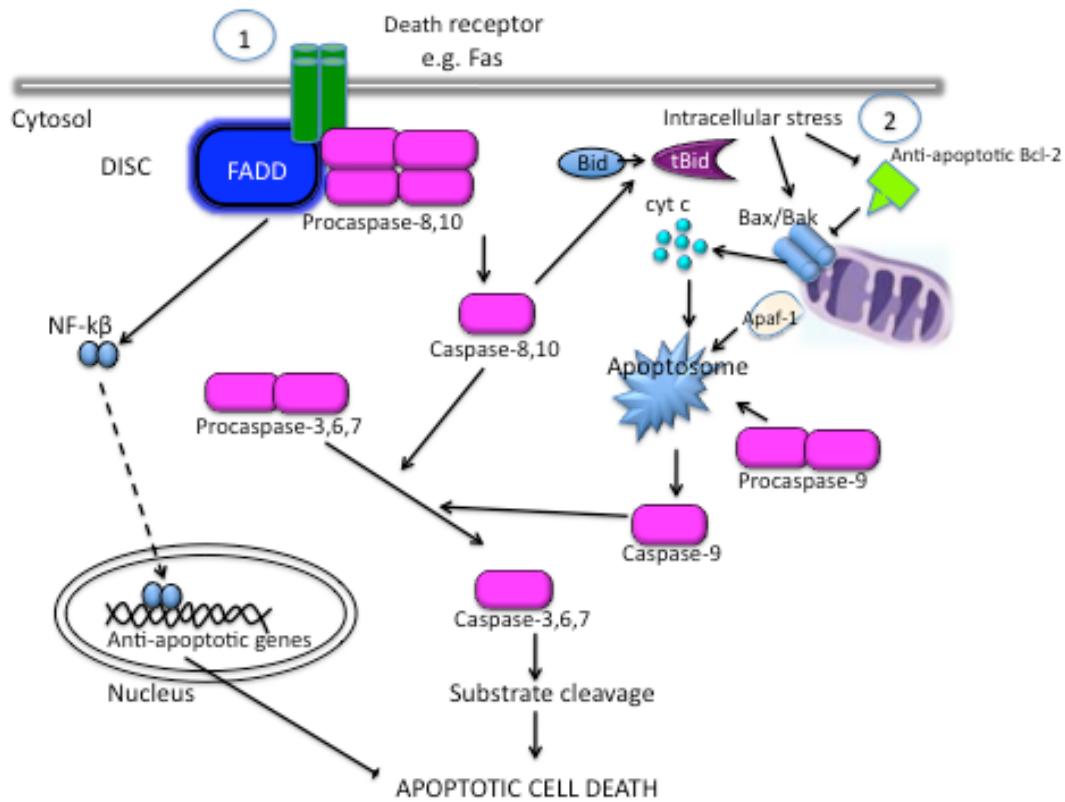
Figure 1.5 Schematic representation of apoptotic signalling.

(1) Extrinsic signalling pathway stimulation is initiated upon stimulation of death receptors (e.g. Fas, TNFR1), a death –inducing signalling complex (DISC) is formed and nuclear factor NFkß activated thus resulting in transcription of anti-apoptotic genes. Fas-associated death domain (FADD) as one of the main components of DISC activates Caspases-8,-10 which subsequently activate Caspases-3,-6,-7 also known as executioner Caspases. Nevertheless, Caspase-8 elucidates the cleavage of anti –apoptotic BCl-2 protein family thus activating the intrinsic pathway (2). (2) Stimulation of the intracellular stress activates Bax/Bac, which results in production of cytochorome c (cyt c). Cyt c predisposes the formation of apoptosome responsible for activation of Caspase-9 and the executioner Caspases-3,-6,-7 (Duprez et al., 2009, Youle and Strasser, 2008). Adapted from Duprez et al., 2009.

Whilst apoptosis is programmed cell death, necrosis, is a consequence of pathologic incident and does not require specific intracellular signalling; therefore it is referred to as non-programmed 'accidental' or 'uncontrolled' cell death. Unlike apoptosis, it does not require activation of specific intracellular signalling (Kühn *et al.,* 2004) and can be characterised as a form of caspase-independent cell death by gain of cell volume, organelle swelling (e.g. mitochondria), followed by loss of membrane integrity which leads to cellular disintegration and random fragmentation of nuclear DNA that occurs following membrane rupture and release of intracellular contents into the extracellular space (Kruz *et al.,* 2004, Duprez *et al.,* 2009, Kaczmarek *et al.,* 2013). Chondrocytes die *via* necrosis after direct mechanical insult or by apoptosis with studies suggesting that necrosis could be triggered *via* the TNFR in the presence of Caspase inhibition thus suggesting that necrosis could also be a cell-mediated form of cell death thereby termed necroptosis (Christofferson and Yuan, 2010, Gong *et al.,* 2013, Kaczmarek, 2013). Numerous mediators are involved in the execution of necrosis (and necroptosis), including reactive oxygen species (ROS), calcium (Ca^{2+}), calpains, cathepsins, phospholipases, and ceramide (Degterev *et al.,* 2005, Vanlangenakker *et al.,* 2012).

Apoptosis has been documented to occur in OA (Goggs *et al.,* 2003), however its cause and contribution to the pathogenesis of the disease are yet to be revealed. A number of stimuli currently presently of interest e.g. nitric oxide (NO), TNF-α, IL-1 and mechanical stress. The effect of NO requires further study but is has variable consequences on apoptosis either prompting or preventing it (Borter, 2004). NO induces AVD and can alter the actin cytoskeleton, with the cytokines IL-1β and IL-6 being able to elevate NO (Lotz, 2003, Borter, 2004, Kurz, 2005). Although NO has been shown to either prompt or prevent apoptosis and can be induced by IL-1β and TNF-α, which are associated with the inflammatory effects observed in OA (Lotz, 2003, Borter, 2004, Kurz, 2005, Goldring and Otero, 2011, Goldring *et al.,* 2012). These cytokines disturb the reconstruction of articular cartilage by elucidating the production of MMPs and aggrecanases (also known as

ADAMTS) that cleave all kinds of ECM proteins, collagen and the PGs network thus limiting the anabolism of the ECM (Goldring *et al.,* 2012, Loser *et al.,* 2012).

NO donors induce chondrocyte apoptosis and increase cyclooxygenase 2 (COX-2) expression stimulating increases in postaglandin E_2 (PGE_2), (Gosset *et al.,* 2008, Loser *et al.,* 2012). Although, the role of PGE_2 is still unclear it has been reported to induce apoptosis in healthy bovine articular cartilage (Goggs *et al.,* 2003, Jeffrey and Aspden, 2007). Furthermore, the presence of pro-inflammatory mediators such as IL-1β and TNF-α found within OA joints and synovial fluid have a significant effect on cartilage metabolism and act directly on chondrocyte catabolism leading to increased expression of MMPs (Sabatini *et al.,* 2000, Gosset *et al.,* 2008) and apoptosis (Blanco *et al.,* 2011, Loser *et al.,* 2012).

Mechanical loading can trigger apoptosis whereby extensive loads lead to cell necrosis as well as structural damage (Chen *et al.,* 2001, Torzilli *et al.,* 2001, Borrelli, 2006, Stolberg *et al.,* 2013) with pressures as low as 4.5 MPa (Loening *et al.,* 2000) inducing apoptosis. Furthermore, it has been shown that mechanical stress can induce a biphasic cell death that is both necrotic and apoptotic. Research by D'Lima and Tew have suggested that initial chondrocyte death induced by mechanical trauma (within the first 6 h) is necrotic as no significant difference was detected in apoptotic cell death, conversely apoptosis has been reported to increase by 30 % at 96 h post mechanical load (D'Lima *et al.,* 1998, Tew *et al.,* 2000).

1.4.2 Mechanical trauma and cell viability

Within articular cartilage, the chondrocyte cytoskeleton is a dynamic three-dimensional (3D) network capable of withstanding mechanical stress (Durrant, 1999, Goldring, 2000, Kurz *et al*., 2005). *In vivo* the levels of stress can be considerable, for example, the physiological load measured in the human femoral head varies between 2-4 MPa, during normal walking (Hodge *et al*, 1986) whilst during running and jumping these levels vary between 20-40 MPa (Hodge *et al*, 1986, Aspden *et al*., 2004, Grodzinsky, 2005). Understanding the physiology and pathology of articular cartilage in order to understand the aetiology of OA is paramount. Animal models of OA have shown that impact loading above the normal range can cause damage to the extracellular matrix (ECM) leading to increased water content (Torzilli *et al*, 1999, Kruz *et al.,* 2001). Cartilage swelling and collagen damage, decreased stiffness (Kruz *et al.,* 2000, Kruz *et al.,* 2001, Chen *et al.,* 2005), loss of glycosaminoglycans (GAGs) (Quinn *et al.,* 1998, Torzilli *et al*, 1999), tissue loss, up and down regulation of gene expression, cell death as well as initiation of cartilage degradation and development of OA (Gulak, 1995, Buckwlter and Mankin, 1997, D'Lima *et al*., 2001a, Bush and Hall, 2005, Aspden *et al*., 2006, Goldring, 2012a).

Mechanical impact or joint overuse has also been reported to be responsible for cartilage fissures and lesions (Figure 1.6; Radin *et al.,* 1981, Donohue *et al.,* 1983, Bush and Hall, 2003, Bush *et al.,* 2005) thus suggesting that mechanical OA could be initiated from repeated impacts. With biochemical and morphological nature of the disease showing a possible 'pre-osteoarthritic state' in relation to the over use induced joint damage (Vasan, 1983, Brown *et al.,* 1984, Lammi *et al.,* 1993, Lequesne *et al.,* 1997). These findings have been further supported with high impact sport including football (Lindberg *et al.,* 1993, Vingared *et al.,* 1993, Roos *et al.,* 1994), weightlifting, running and boxing (Kujala *et al.,* 1994, Kujala *et al.,* 1995), displaying an increased risk of OA in knee and hip joints.

(A)   (B)
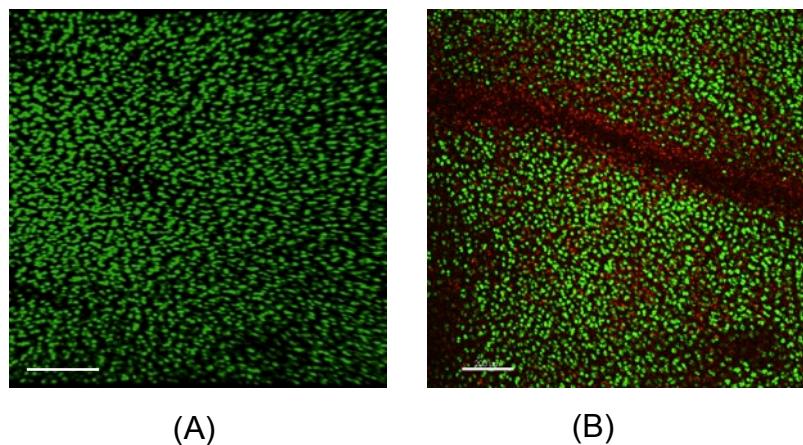
Figure 1.6 Overview of chondrocyte death arising from the application of single impact load to bovine cartilage.

(A) Healthy non impacted bovine cartilage tissue.

(B) Single injurious impact damaged the cartilage by causing fissures and lesions to occur. Green spots represent live cells, red spots represent dead cells images were acquired by axial confocal laser scanning microscopy (CLSM) projections imaging the superficial zone of cartilage 'top-down' from the articular surface. Bar = 100 µm

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Acute mechanical traumatic joint injury in terms of sport injury or traffic accident is thought to be one of the factors in the development of secondary OA (Borrelli, 2006). Articular cartilage has poor repair potential and generally does not repair (Buckwalter and Mankin 1997, Newman, 1998, Grodzinsky, 2005) whereby acute traumatic lessons may occur as a consequence of intra-articular fracture and high-impact injury, or following ligament injury, and are believed to be factors that lead to the development of secondary OA (Hunzinker, 2002, Mankin *et al.,* 2000, Borrelli, 2006). Other factors include inflammation, genetic predisposition, female gender, obesity, or occupational hazards (Davis *et al.,* 1989, Kurz, 2005, Buckwalter, 2006, Goldring and Marcu, 2009, Iannone and Lapaduda, 2010, Goldring and Otero, 2011).

OA is a degenerative disease with well-known final stages however, the early stages and the triggers of the degenerative pathway are yet to be elucidated. The disease is characterised by progressive degeneration of articular cartilage leading to chronic joint pain and restrictions of joint function in the affected joints (Buckwalter, 1995, Aspden, 2006, Richter and Lorenz, 2006), accompanied with sclerosis and remodelling of subchondral bone and formation of cysts (Johnston, 1997, Buckwalter, 2006). Nevertheless, the only current treatment is arthroplasty and the mechanism of the progression of the disease is still unknown.

Over the last decade, several animal models have been established to investigate the mechanism of mechanical overload and injury (Kruz *et al.,* 2005, Amin *et al.,* 2011, Poulet *et al.,* 2011). Single impact loading (simulated by using drop tower) is a research technique used to study the mechanism of chondrocyte cell death as a model of cartilage damage by utilizing, various heights and weights (Bush and Hall, 2005, Aspen 2006, Huser and Davis 2006, Jeffrey, 2006, Vertramo and Sheedhom, 2007, Henson and Vincent 2008, Martin *et al.,* 2009, Yeow *et al.,* 2010, Henson *et al.,* 2012). Chondrocyte cell death has a major impact on degeneration of articular cartilage and is dependent on strain rate and mechanical load (Aspden *et al.,* 2006).

Mechanical injury has been found to influence the biosynthetic activity of articular cartilage using numerous models to simulate mechanical overload (Bush *et al.,* 2005, Jeffrey and Aspden, 2006, Huser and Davis 2007, Henson and Vincent 2008, Martin *et al.,* 2009, Henson *et al.,* 2012). Importantly, it is recognised that injury does not only influence the biosynthetic activity directly but can also influence the chondrocytes ability to respond (Henson *et al.,* 2012). Studies suggest that chondrocyte death and viability are closely linked to ECM integrity and induce high levels of cartilage injury resulting in cell death (necrosis, apoptosis, or both) and release of PG (Loening *et al.,* 2001, Lotz, 2004, Bush *et al*., 2005, Grodzinsky *et al.,* 2005, Martin *et al.,* 2009). Previous studies utilising mechanical models of *in situ* chondrocytes showed that the key effect on morphology is cell volume, decreases in cell volume renders the cell less susceptible to mechanical load. Conversely, swelling of *in situ* chondrocytes by reduced osmolarity elevates their sensitivity to mechanical load and increases cell death (Bush and Hall, 2003, Bush *et al*., 2005, Amin *et al.,* 2008).

AIMS

The aim of this study is to determine the role of single mechanical impact injury in the first stages of post mechanical trauma, the role that the actin cytoskeleton and $[Ca^{2+}]_i$ play and identification of a novel chondro-protective agent following single impact load injury.

CHAPTER 2

2. MATERIALS AND METHODS

2.1 Materials

2.1.1 Tissue and Cell Culture

NAME	COMPANY	PRODUCT CODE	WORKING CONCENTRATION
Dulbecco's Modified Eagle Medium (DMEM)	Sigma-Aldrich, Poole, UK	D6171	N/A
Penicillin / Streptomycin Solution	Sigma-Aldrich, Poole, UK	P0781	1 %
Sodium Chloride	Fisher Scientific, Loughborough, UK	S13160/65	Varied; depending upon the experimental protocol
Trypsin-EDTA	Invitrogen, Paisley, UK	25300	0.05 %
Spirit, Methylated	VWR, Lutterworth, UK	302444E	N/A
Collagenase	Sigma-Aldrich, Poole, UK	C0773	0.08 g
Trypan Blue	Sigma-Aldrich, Poole, UK	T8154	0.4 %

Table 2.1 Reagents for tissue culture.

2.1.2 Fluorophores used

NAME	COMPANY	PRODUCT CODE	WORKING CONCENTRATION
Calcein-AM	Anaspec Inc, Freemont, USA	89201	5 μM
Alexa 488 (conjugated to phalloidin)	Invitrogen, Paisley, UK	A12379	35 nM
Propidium Iodide	Invitrogen, Paisley, UK	P1304MP	1 μM
Fluo-4, AM	Invitrogen, Paisley, UK	F14201	2 μM

Table 2.2 Fluorophores used for CLSM.

2.1.3 Cytoskeletal Studies

NAME	COMPANY	PRODUCT CODE	WORKING CONCENTRATION
Ammonium Chloride	Sigma-Aldrich, Poole, UK	A0171	50 mM
Bovine Serum Albumin	Sigma-Aldrich, Poole, UK	A2153	1 %
Glycine	Fisher Scientific, Loughborough, UK	BPE381	100 mM
Para-formaldehyde	Sigma-Aldrich, Poole, UK	533998	4 %
Phosphate Buffer Saline	Fisher Scientific, Loughborough, UK	BR0014G	N/A
Triton x100	Fisher Scientific, Loughborough, UK	ZT3751	0.5 %
Tween 20	Fisher Scientific, Loughborough, UK	BPE337	0.05 %

Table 2.3 Reagents used to study actin cytoskeleton.

2.1.4 Molecular Biology Reagents

NAME	COMPANY	PRODUCT CODE	WORKING CONCENTRATION
Nucleospin®	MACHERY-NAGEL, Düren, Germany	740 933	N/A
Absolute Ethanol	VWR, Lutterworth, UK	10107	70 %
Agarose	Fisher Scientific, Loughborough, UK	BP1356	2 %
Quick-Load® 100 bp DNA Ladder	New England Biolabs, Hitchin, UK	N0467S	N/A
GoTaq® Green Master Mix	Promega Corporation, Southampton, UK	M7112	N/A
ImProm-II™	Promega Corporation, Southampton, UK	A3800	N/A
2-Mercaptoethanol	Fisher Scientific, Loughborough, UK	BP176	2 %

Table 2.4 Reagents used for RNA extraction, reverse transcription of RNA and cDNA PCR.

NAME	COMPANY	PRODUCT CODE	WORKING CONCENTRATION
NuPAGE® 10 % Bis-Tris Gel	Invitrogen, Paisley, UK	NP0301	N/A
NuPAGE® Running Buffer (20x)	Invitrogen, Paisley, UK	NP0001	N/A
NuPAGE® Transfer Buffer (20x)	Invitrogen, Paisley, UK	NP0006	N/A
NuPAGE® LDS Buffer (4x)	Promega Corporation, Southampton, UK	NP0007	N/A
Full Range Rainbow^{TM} Weight Marker	GE Healthcare, Amersham, UK	RPN800	N/A
Pierce®ELC Western Blotting Substrate	Fisher Scientific, Loughborough, UK	10322094	N/A
Kodak Developer and replenisher	Kodak, Hemel Hempstead, UK	190 0943	N/A
Kodak Fixer and replenisher	Kodak, Hemel Hempstead, UK	190 1875	N/A
Hot Lysis Buffer	See Appendix III	N/A	N/A
TBST Buffer	See Appendix III	N/A	N/A
Methanol	Fisher Scientific, Loughborough, UK	N/A	N/A
Skimmed milk	Sainsbury's, London, UK	N/A	5 %

Table 2.5 Western blot reagents.

NAME	COMPANY	PRODUCT CODE	WORKING CONCENTRATION
Monoclonal Anti-α-Tubulin CloneB-5-1-2	Sigma-Aldrich, Poole, UK	T5168	1:2000
Polyclonal Goat Anti-mouse Immunoglobullins/ HRP	Dako Cytomation, Glostrup, Denmark	DK-2600	1:2000
Anti-rabbit IgG, HRP-linked Antibody	Cell Signalling Technology, Danvers, USA	7074	1:2000
P-Cofilin Antibody	Cell Signalling Technology, Danvers, USA	3313S	1:2000
Cofilin Antibody	Cell Signalling Technology, Danvers, USA	5175S	1:2000
Profilin-1 Antibody	Cell Signalling Technology, Danvers, USA	3237	1:2000
Gelsolin	Abcam, Cambridge, UK	Ab74420	1:2000

Table 2.6 Antibodies used.

2.1.5 Pharmacological Reagents

NAME	COMPANY	PRODUCT CODE	WORKING CONCENTRATION	REFERENCES
Dimethyl Sulphoxide	Fisher Scientific, Loughborough, UK	D4121	0.1 %	-
Ethylene glycol tetraacetic acid (EGTA)	Sigma-Aldrich, Poole, UK	E0396	2 mM	(Erickson *et al*., 2000, Kerrigan and Hall, 2008)
Gadolinium (III) chloride	Sigma-Aldrich, Poole, UK	G7532	100 μM	Kerrigan and Hall, 2006)
REV5901	Sigma-Aldrich, Poole, UK	R5523	50 μM	(Bush and Hall, 2001a, Musser *et al*., 1987, Van Inwegen *et al*., 1987
REV5901 *para*-isomer (L-655,238)	Enzo® Life Sciences, Exeter, UK	BML_EI210_0010	50 μM	(Bush and Hall, 2001a)
Jasplakinolide	Invitrogen, Paisley, UK	J7473	5 μM	(Bubb *et al.,* 1994)
Wortmannin	Sigma-Aldrich, Poole, UK	W1628	10 nM	(Starkman*, et al.,* 2005)
Uridine	Sigma-Aldrich, Poole, UK	U6381	100 µM	(Koolpe *et al*., 2005)
Lantraculin B	Sigma-Aldrich, Poole, UK	L5288	10 μM	(Coue *et al*., 1987, Kerrigan and Hall, 2005)

Table 2.7 Reagents used.

2.1.6 Biochemical and Experimental Solutions

Cartilage explants were isolated in serum-free Dulbecco's Modified Eagle Medium (DMEM) containing penicillin (100 units.ml^{-1}) and streptomycin (50 µg.ml^{-1}) pH 7.4; 280 mOms, Calcein-AM (5 µM), propidium iodide (1 µM) and Alexa phalloidin (5 µM) were used to visualise chondrocytes by Confocal Laser Scanning Microscopy (CLSM). These fluorescent indicators were used in stock solutions prepared in dimethylsulfoxide (DMSO), deionised distilled water or methanol, respectively.

Filter-sterilised 50 mM sodium chloride (NaCl) was used for the preparation of hypertonic DMEM (various osmotic challenges) and osmolarity was measured using a Vapro^{TM} vapor pressure osmometer. A stock solution of gadolinium chloride (GdCl$_3$), 10 mM was prepared in water, REV 5901 was reconstituted into DMSO to a working concentration of 50 µM, L-665,238 reconstituted into DMSO to a working concentration of 50 µM, wortmaninn, uridine also reconstructed in DMSO with working concentration of 10nM and 100 µM.

In experiments that involved the removal of extracellular calcium ([Ca^{2+}]$_0$) a basic physiological saline (BPS) was used containing: glucose (10 mM), potassium chloride (KCl; 5 mM), magnesium chloride (MgCl$_2$; 1 mM), HEPES (1 mM) and ethylene glycol tetraacetic acid (EGTA; 2 mM). For a paired control the above solution was used whereby the EGTA was substituted by the addition of calcium chloride (CaCl$_2$; 1 mM). All solutions were adjusted to a pH of 7.4 ± 0.05 using sodium hydroxide (NaOH). The osmolarity of all solutions was measured using a Vapro^{TM} vapour pressure osmometer and adjusted by the addition of NaCl to +/- 5 Osm of the desired osmolarity.

2.2 Methods

2.2.1 Bovine dissection and isolation of explants and primary chondrocytes

Bovine articular cartilage was removed from load-bearing regions of the metacarpal-phalangeal joints of 18-21 months old female animals on the day of slaughter (obtained from local abattoir with ethical permission and used within 3 days). The hoof and skin were removed before the exposure of the joint capsule (Figure 2.1), cartilage explants, without subchondral bone, were removed under aseptic condition and placed in 280 mOsm DMEM, supplemented with 1 % penicillin and streptomycin. Fresh scalpels were used to minimise cell damage as previously described and cartilage explants were allowed to recover overnight (Bush and Hall, 2005, Kerrigan and Hall, 2005, Amin *et al.,* 2010).

In order to obtain primary chondrocytes cartilage explants were excised from bovine legs using several animals and pooled chondrocytes were isolated from explants by overnight incubation (18-20 h) with collagenase (Sigma-Aldrich, Poole, UK; 0.8 mg ml^{-1} DMEM). Cells were kept in 380 mOsm DMEM whereby the osmolarity of the DMEM was raised by the addition of NaCl to prevent chondrocyte swelling due to their release from the matrix (Hall, 1996). The digest was then passed through a 20 µm nylon filter and washed 3x by centrifugation (8 min, 500g) and chondrocytes were cultured. Absolute cell number used for PCR experiments was 5×10^6 cells, 1×10^6 cells for Western blot analysis and CLSM examinations were performed at 1×10^4 cell number.

Figure 2.1 Cartilage removal.

Full depth explants were removed from the apical surface of metacarpal joint of bovine animals under aseptic conditions. Explants were allowed to rest at 37° C and 5 % CO_2 in DMEM for overnight (Bush and Hall, 2001a).

2.2.2 Impact Tower and Calculation of Impact Energy

In order to investigate the effect of blunt trauma on articular cartilage cells individual explants were subject to a single impact trauma by using a 'drop tower' following a previously reported protocol (Gregory *et al.,* 1995, Jeffrey *et al*., 1995, Bush *et al.,* 2005). The drop tower, (Figure 2.2) was constructed of three vertical parallel steal rods in which an impactor could fall freely with minimum friction therefore, a known mass could be dropped onto a sample of cartilage from a known height (Jeffrey *et al*., 1995, Bush and Hall, 2005).

The impact force pressure was calculated assuming linear acceleration under gravity and it was calculated that an impact force pressure of 1.34 N is the result of known mass (134 g) and height (10 cm) of the 'drop' carried out on the cartilage sample (Table 2.8). Following a single impact, cartilage explants were removed from the drop tower and placed flat on a dish and viewed at a perpendicular angel to the synovial (articular) surface. To insure that the tissue was prevented from moving during the image acquisition process, cyano-acrylic glue (Bostik Ltd, Leicester, UK) was applied to one edge of the sample.

The values above are significantly lower compared to values published in the literature regarding *in vivo* joint forces but these values have been optimised to allow for studying cell death as a result of a blunt trauma for articular cartilage explants, as they do not tend to destroy the tissue thus giving the opportunity to measure cell death. In addition, articular cartilage within an *in vivo* environment would be supported by bone and protected by synovial fluid thus allowing cartilage to withstand greater forces. However, the impact tower model is not designed to be an exact model for mimicking the *in vivo* process but is a suitable model to study the effects of blunt mechanical trauma on articular cartilage (Jeffrey *et al.,* 1995).
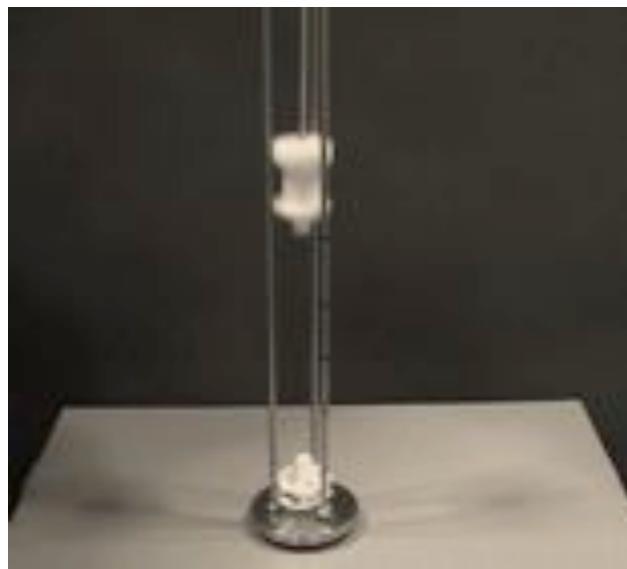
Figure 2.2 Drop tower for cartilage impact.

The tower was constructed of three rods, impactor (shown in motion) and the base of the tower are made of hard nylon polished to a smooth finish (I would like to thank Dr. Andrew C Hall at the University of Edinburgh for the use of the drop tower).

MASS (g)	DROP HEIGHT (cm)	IMPACT VELOCITY (m/s)	ENERGY (J)	FORCE (N)	PRESSURE (MPa)
134	5	0.99	0.070	0.070	4×10^{-3}
134	10	1.40	0.132	1.32	7.5×10^{-3}
134	20	1.98	0.263	0.263	15×10^{-3}

Table 2.8 Impact energy and velocity corresponding to mass and height.

Values were calculated using physics equations and they are theoretical where by, force was calculated from the known mass and the acceleration (F=ma). Pressure was calculated from the force and the base ('drop') area (1.77 cm^2) of the impact tower.
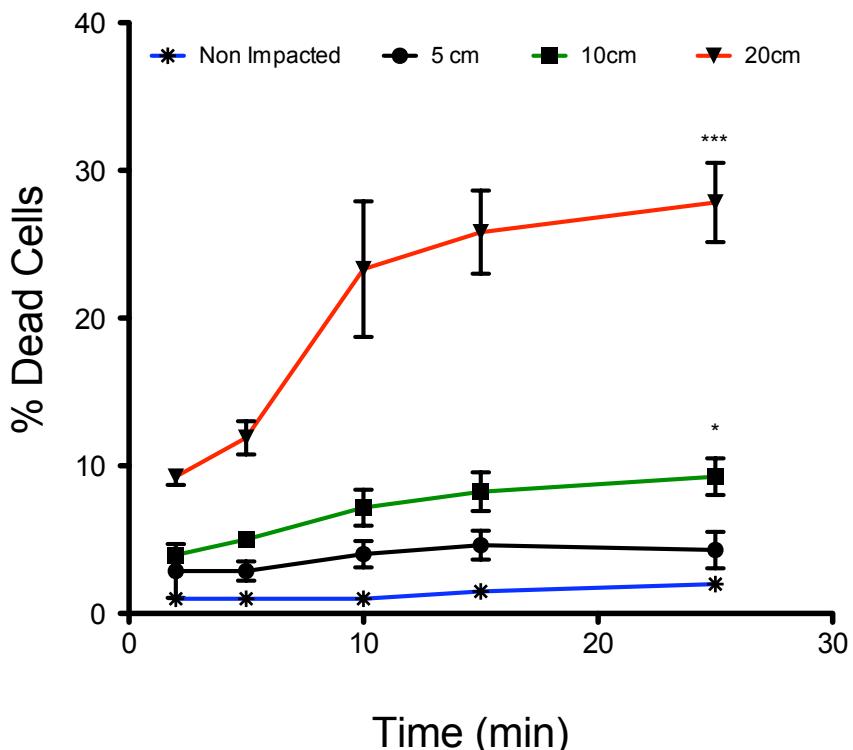
Figure 2.3 Cell response post impact from different heights.

Full depth cartilage explants were subject to a single impact by using the impact tower. Non-impact and impact (5-20 cm) tissue using 134 g was used and cell viability determined by CLSM. Explants were incubated with 5 µM calcein-AM and propidium iodide 1 µM prior to visualisation and samples were imaged at 2, 5, 10, 15 and 25 min post impact. The number of dead cells was expressed as percentage of the total cell number. Data are mean +/- SEM, N=6 from 3 distinct experiments. Statistical analysis (Student's unpaired t-tests) were given as *p< 0.05, **p< 0.01 and ***p< 0.001 all versus 5 cm impact.

2.2.3 Preparation of Tissue and Primary Cells for Confocal microscopy

Confocal Laser Scanning Microscopy (CLSM) is a technique that has found tremendous utility in *in vitro* three-dimensional imaging (3D). Confocal microscopy provides an opportunity to collect high-resolution, 3D images that follow dynamic cellular changes over periods of time (Semwogerere and Weeks, 2005, Kerrigan and Hall, 2008). The fundamentals of CLSM date back from 1955 when Marvin Minsky, a Fellow from Harvard University, reported a point-by-point image construction by focusing a point of light sequentially across a specimen and then collecting some of the reflecting rays (Leica, 2004, Semwogerere and Weeks, 2005). Confocal microscopes use a laser light source focused onto a focal point of the specimen surface being imaged by the objective lens. Photons are emitted from the fluorophore, used to stain the specimen and collected through the objective separated into the detection aperture via a 'pinhole'. The 'pinhole' is the critical part of the CLSM where by out-of-focus light rays are obstructed, thus creating very clear and sharp images within a 3D space.

The ability of confocal microscopes to create sharp images of optical sections makes possible the construction of 3D images by assembling the data gathered from a series of successive focal panels (termed a z-stack), which can be used to investigate changes in cell volume, cell survival and actin cytoskeleton. Full depth explants and freshly isolated cells were visualised using an upright Leica SP2 CLSM (Leica Microsystems, Milton Keynes, UK) and image acquisition collected as previously described (Bush *et al.,* 2005, Kerrigan and Hall, 2008). Images acquired on the CLSM were reassembled and analysed on Imaris software 7.4 (Bitplane, Zurich, Switzerland). All images were acquired at a resolution of 1024x1024 pixels using an optimal pinhole radius of 1 Airy unit, which is defined as the diameter of the Airy disk for each given objective (Semwogerere and Weeks, 2005, Ramshesh and Lemasters, 2008). Simply, an Airy disc is a diffraction pattern created by the sample under the microscope and is another way to describe the resolution of the microscope. By adjusting the pinhole diameter

of the Airy disc, or 1 Airy unit signal to noise ratio of the light from the sample is high and thus resulting in optimal acquisition.
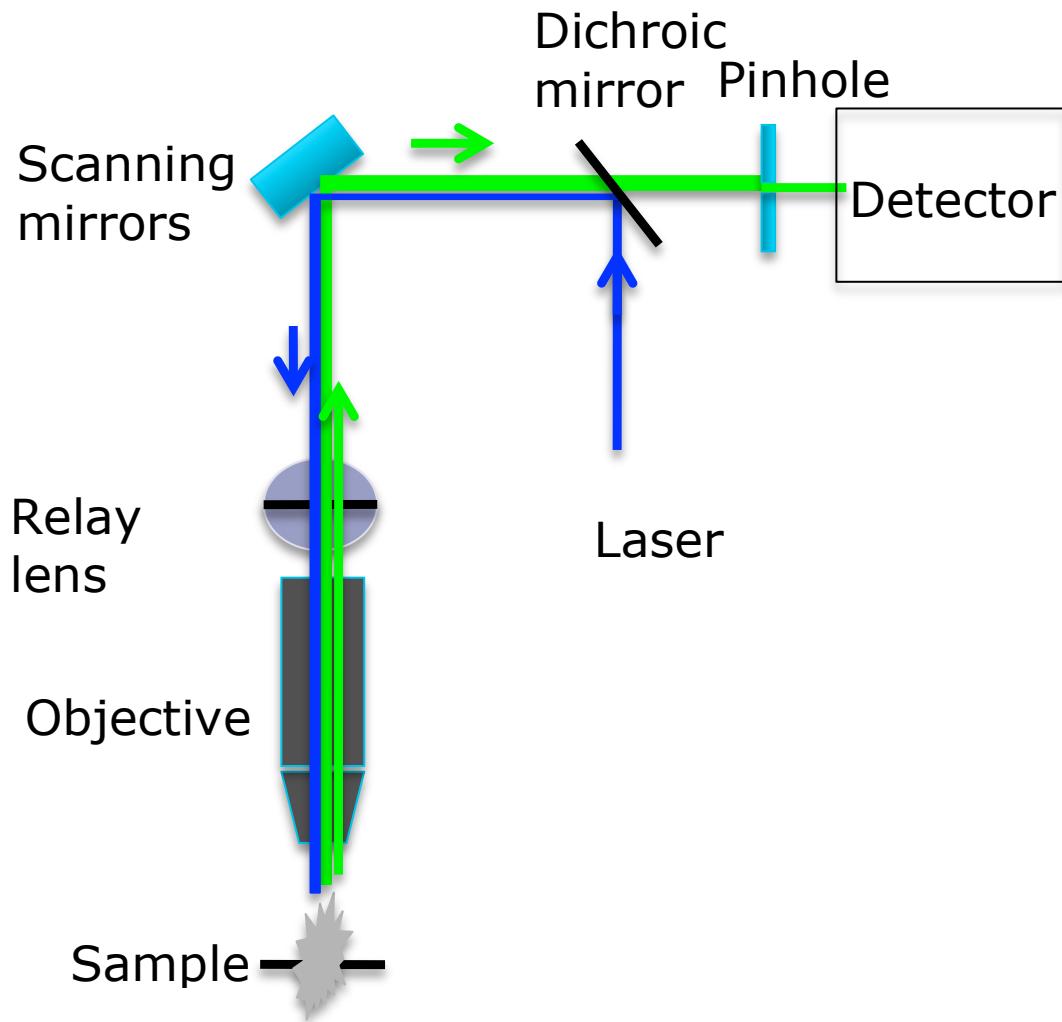
Figure 2.4 Confocal Microscope Optical Path.

Confocal microscopes use a laser light source that is focused onto a focal point of the specimen surface being imaged by the objective lens. Adsorbance of excited light results in the release of an emitted photon reemitted from the fluorophore used to stain the specimen and collected through the objective separated into the detection aperture by the beam splitter and passed through the 'pinhole', this obstructs 'out-of-focus light rays, thus creating very sharp images. The light signal coming from the specimen is recorded into a detector (photomultiplier tube) that converts light into electrical signal able to be interpreted by computer.

2.2.3.1 Preparation of Tissue for Imaging cell survival following Impact

Prior to experimentation, cartilage explants were incubated with Calcein-AM to determine living cells and propidium iodide (PI) for dead/dying cells (30 min, 37° C, 5 µM and 1 µM, respectively). Calcein-AM is a membrane-permeant and non-fluorescent but, upon cell entry in leaving cells, the AM-group is cleaved by intracellular esterases and the fluorophore calcein is released in an impermanent form (Grynkiewicz *et al.,* 1985). Conversely, PI is a membrane impermanent dye generally excluded from live cells and as such is only able to bind to DNA (the target molecule), when the cell membrane becomes permeable; a process that occurs early during cell death (Bush *et al.,* 2005).

Following impact, explants were attached to a 22 mm dish with cyano-acrylic glue (Bostik Ltd, Leicester, UK) and fresh 280 mOsm DMEM added and the plastic dish placed onto a heated stage (37° C) of an upright Leica SP2 CLSM (Leica Microsystems, Milton Keynes, UK). Images were acquired using a x10 dry objective lens, at a resolution of 1024 x 1024 pixels with calcein excited at 488 nm (argon laser; Figure 2.5) and the emission recorded through a bandpass filter 510-525 nm. Propidium iodide excited at 543 nm (He-Ne laser; Figure 2.5) with the emission recorded through a bandpass filter 660-709 nm. For the generation of 3D images a z-step of 5.0 µm was used along the Z-axis. To eliminate the potential effect of 'bleed-through' (overlap between emission spectra) between calcein and PI that could lead to false images of samples stained with both fluorophores sequential imaging was used. The technique obstructs 'bleed-through' by the consecutive excitation of the fluorophores instead of simultaneous excitation thus ensuring that the image taken is solely from the emitting fluorophore and not from the second fluorophore (Leica 2006, Ramshesh and Lemasters, 2008).

2.2.3.2 Preparation of Freshly Isolated explants or Primary Cells for Imaging Cell volume and morphology

Freshly isolated explants/cells were prepared at a low density 1×10^4 into 22 mm dishes and incubated with Calcein-AM (30 min, 37° C; 5 µM) and the excessive, non-incorporated fluorophore removed by washing with 280 mOsm fresh DMEM. Dishes were subsequently placed onto a heated stage (37° C) of the CLSM and images acquired using a x63 immersion objective lens (NA=0.63), at a resolution of 1024 x 1024 pixels. 3D images were generated with z-stack series acquired using 1.0 µm intervals along the z-axis.

2.2.3.3 Preparation of Tissue or Primary cells for cytoskeletal staining

To investigate the effects of impact trauma on actin cytoskeleton organisation a modified, method previously described, was used (Kerrigan and Hall, 2005). Cartilage tissue/cell cultures were fixed with 4 % ice-cold paraformaldehyde (PFA) for 30 min at room temperature and then washed 3X with ice-cold PBS to remove the excesses PFA. Actin labelling was based on the Triton method using the fluorophore Alexa 488-phalloidin. Samples were quenched for 10 min in ice-cold NH_4Cl followed by subsequent permeablisation using 0.1 % Triton X100 in PBS for 5 min under gentle agitation, rinsed in 100 mM glycine to stop the permeabilisation and washed 3x with PBS before being blocked with 1 % bovine serum albumin (BSA) and 0.05 % Tween-20 in PBS for 30 min. F-actin was labelled with Alexa 488-phalloidin (5 µl/ml) under gentle agitation for 45 min in the dark. After labelling, excessive dye was removed by washing 3X in ice-cold 0.05 % Tween-20 in PBS and stored at 4^0 C until required for imaging; within 24 h. The fluorophore Alexa 488-phalloidin was excited using a 488 nm laser (Argon-laser; Figure 2.5) and images acquired using a x63 water-emersion objective at samples 1024 x 1024 pixels, with a z-step of 0.3 µm. To reduce the signal to noise ratio average of 4x zoom was used for every cell.
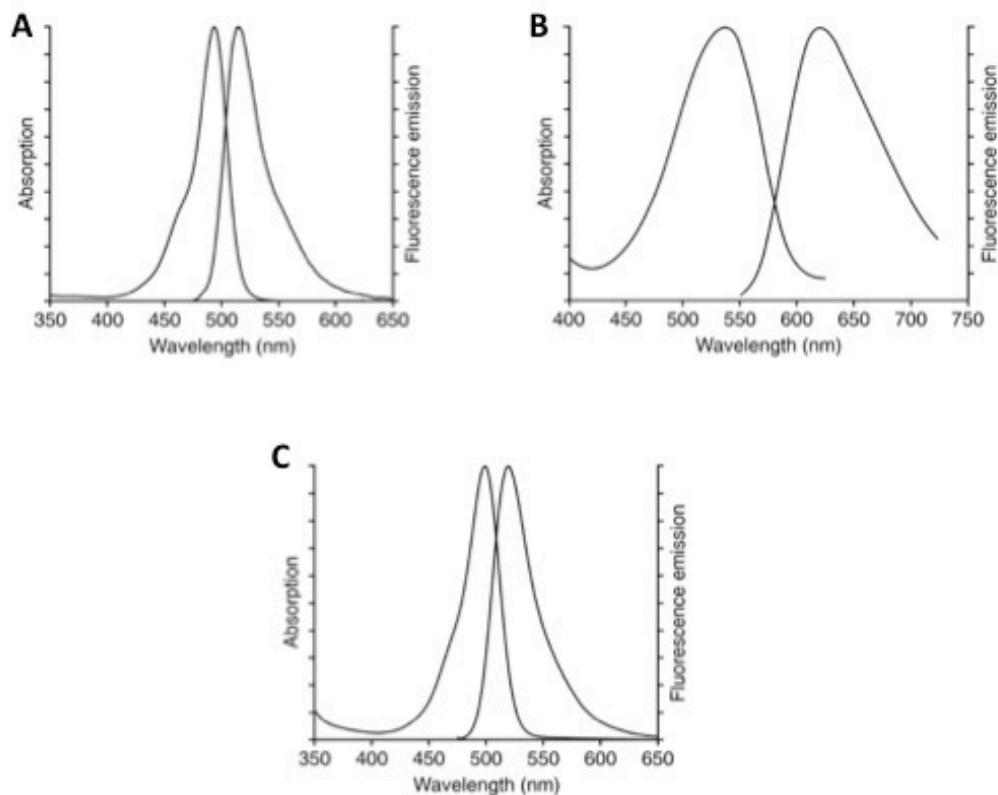
Figure 2.5 Excitation and Emission spectra for Calcein-AM, Propidium Iodide (PI) and Alexa Fluor 488 Phalloidin.

Excitation spectra for the fluorophores used wavelengths of 488 nm for both Calcein and Alexa 488 phalloidin (A&C), and 543 nm for PI (B). Emissions optimal pass band was observed to be 510-590 for Calcein and 570-710 for PI thus presenting a possibility for bleed-through effect that indicated the need of sequential imaging. Alexa flour 488 phalloidin demonstrated a pass band of 535-590 nm. Figure adapted from Sigma-Aldrich documentation.

2.3 Cell shrinkage and Ca^{2+}

To measure changes in intracellular calcium ($[Ca^{2+}]_i$) in response to a change in osmolarity a FLUOstar OPTIMA plate reader (BMG Labtech, Aylesbury, UK) equipped with 320 nm and 520 nm excitation and emission lenses, was used. Solutions of varying osmolarity were prepared and media osmolarities were measured with Vapro^{TM} vapour pressure osmometer, with all solutions prepared to ± 5 mOsm. Cells were plated onto a 12 well plate (low density), incubated with 2 µM of Fluo-4, 30 min and kept at 37°C for the duration of the experiment. Due to the nature of the fluorophore any dye that was not specifically associated with the cell surfaces was removed (e.g. DMEM has red colour) and the measurements were taken in PBS. Through the instrument's liquid-handling system an osmotic gradient (10-90 %) was applied 55 s after the beginning of each recording and subsequently for the following 300 s. Data were acquired every 15 s and subsequently transferred to Microsoft Excel for analysis. All experiments were performed within 12 h to ensure absence of extracellular matrix formation as well as rounded cell shape.

2.4 Molecular biology

2.4.1 Polymerase chain reaction (PCR)

2.4.1.1 RNA purification and cDNA conversion

Total RNA was extracted from freshly isolated primary bovine chondrocytes using a commercially available kit (Nucleospin RNA II Kit, Macherey-Nagel, Düren, Germany). Briefly, according to the manufactures protocol the lysate from 5 x 10^6 cells was filtered and 350 µl of RP1 buffer together containing 3.5 µl ß-mercaptoethanol (ß-ME) was added to the sample following a vigorous homogenisation through a 21-gauge needle (5-10 times) and mixed with 350 µl of 70 % ethanol. Samples were then transferred into a Nucleospin silica column and RNA allowed to bind to the column and spun for 30 s at 11,000 x g using a bench top centrifuge (MiniSpin®, Eppendorf, Cambridge, UK). The flow-through was discarded and SPM rDNAsa was applied to the reaction mixture thus allowing the elution of highly pure RNA.

The flow-through was discarded and column washed with 200 µl RA2 Buffer containing guanidine salt. The column was then washed twice with 600 µl RA3 buffer and 250 µl of RA3 buffer containing 95 % ethanol to remove salts, the membrane was then spun dry to ensure that no liquid remained. Elution of RNA was carried out in 40 µl of RNase free water and samples kept on ice to be used immediately to make complementary DNA (cDNA).

The RNA concentration was determined by measuring absorbance at 260 nm by NanoDrop-1000 (Fisher Thermo Scientific, Loughborough, UK) and cDNA was synthesized from 1 µg of the total RNA using Improm II Reverse Transcription system (Promega, Southampton, UK) according to manufactures instructions. Briefly, to ensure denaturation of RNA secondary structure, total RNA together with the oligonucleotides were incubated at 70° C for 5 min followed by cooling to 4° C for 5 min to insure hybridization. The next step was to incubate reaction buffer, $MgCl_2$, dNTPs and Reverse Transcriptase (RT) (Table 2.9) 60 min at 42° C at the end of this period RT was inactivated at 70° C for 15 min and the template DNA ready for PCR.

(A)

COMPONENT	CONCENTRATION
Experimental RNA	1 µg per reaction
Random primer	0.5 µg per reaction
Nuclease free water	x

(B)

COMPONENT	VOLUME (µL)
Nuclease-Free Water (to a final volume of 15µl)	x
ImProm-II™5X Reaction Buffer	4.0
$MgCl_2$ (final concentration 1.5–8.0 mM)	2.0
dNTP Mix (final concentration 0.5 mM each dNTP)	1.0
Recombinant RNasin®Ribonuclease Inhibitor	0.5
ImProm II™ Reverse Transcriptase	1.0

Table 2.9 Target RNA preparation (A) and reverse transcription (B).

2.4.1.2 PCR and Gel electrophoresis

PCR was performed on the cDNA synthesized by the above protocol; GoTaq® Green Master Mix (Promega, Southampton, UK) and the following programme were used to synthesize the desired products (Table 2.10 A & B and Table 2.11). Cycles of denaturation (at 94° C), annealing and DNA synthesis were repeated 35 times.

(A)

COMPONENT	VOLUME (μL)
GoTaq Green Mastemix, 2x	12.5
Forward Primer, 10 μl	2.0
Reverse Primer, 10 μl	2.0
cDNA Template	2.0
Nuclease –Free H_2O	6.5
Final Volume	**25**

(B)

PCR STEP	TIME (MIN)	TEMPERATURE (^{0}C)
Initial denaturation	2	94
Denaturation	1	95
Annealing	1	58-62
Elongation	2	72
Final Elongation	5	72
Soak	∞	4

Table 2.10 PCR sample preparation (A) and program condition (B).

GENE	SEQENCE	PRODUCT SIZE	ANNEALING TEMPERATURE (^{0}C)
GAPDH Forward Reverse	5'-AGAACGGGAAGCTTGTCATCTC-3' 5'-TGAGCTTGACAAAGTGGTCGT-3'	743 bp	58
COFILIN Forward Reverse	5'-TGGGGTCATCAAAGTGTTCA-3' 5'-TTGTCTGGCAGCATCTTGAC-3'	300 bp	58
PROFILIN Forward Reverse	5'GACTGCCAAGACGCTAGTCC-3' 5'-ATGTGTGTGGGAAGGAGAGG-3'	300 bp	62
GELSOLIN Forward Reverse	5'-TGCAGCTGGATGACTACCTG-3 5'-GAAGCTCTCCCAGGACACAG-3'	300 bp	62

Table 2.11 Primers and products.

10 μl of the marker (QuickLoad 100 base pairs DNA ladder, New England Biolabs, Hitchin, UK) and 25 μl of the PCR samples were loaded onto 2 % agarose gel (Fisher Scientific, Loughborough, UK) and run in Tris-Borate-EDTA buffer (TBE) (Sigma-Aldrich, Poole, UK). The gel was run at 100 V for ~40 min (5 V/cm) then transferred from the electrophoresis block into a solution of ethidium bromide and soaked there for 30 min washed twice with water and quantified by the ethidium bromide fluorescence, and analysed using ImageJ$^{®}$ software (National Institute of Health, Bethesda, USA).

2.4.2. Western blot

2.4.2.1 Cell lysate Preparation

The lysate was prepared from 1 x 10^6 primary cells that were washed twice in phosphate buffered saline (PBS). Cells were lysed with Hot Lysis Buffer consisting of 10 % v/v Glycerol (Fisher Scientific, Loughborough, UK), 62.5 mM Tris-HCL pH6.8 (Fisher Scientific, Loughborough, UK), 2 % sodium dodecyl sulphate (SDS) (BDH Biochemical, Poole, UK), 100 mM dithiothreitol (DTT) (Sigma-Aldrich, Poole, UK). Proteins were denatured for 5 min at 100 ^{0}C and passed through 25-gauge needle (5-10 times). Extracted proteins were then quantified using NanoDrop-1000 (Fisher Thermo Scientific, Loughborough, UK).

2.4.2.2 Sample preparation and SDS-polyacrylamide gel electrophoresis (-PAGE)

The same vertical NuPage apparatus (Invitrogen, Paisley, UK) was used for electrophoresis and protein blotting. Samples of 50 µg were mixed with mixture of 4x Sample Loading Buffer containing ß-ME (5 μl), the volume of the sample to be loaded to the gel was determined from the protein concentration obtained. 10 μl of Full Range Rainbow^{TM} Weight Marker (GE Healthcare, Amersham, UK) was added to the first well and samples were added to the other wells. The gel was run in 1x running buffer (NuPAGE® Running Buffer (20x) diluted with distilled water) at 200 V constant for 50 min.

2.4.2.3 Transfer of protein to nitrocellulose

Sponges, filter paper and nitrocellulose membrane (Hybond ECL) were soaked in 1x blotting transfer buffer (750 ml distilled water, 200 ml Methanol and 50 ml NuPAGE® Transfer Buffer (20x)). Once the gel had finished running it was removed from the plastic container and the stacking gel was cut out. The gel was positioned on the top of the filter paper and the layers in the transfer cassette were arranged as follows:

- Cassette
- 2x Sponge
- 2x Filter paper
- Gel
- Membrane
- 2x Filter paper
- 2x Sponge
- Cassette cover

All precautions were taken to remove the bubbles that could have appeared in the process of the 'sandwich' blot preparation. Blotting transfer buffer was then poured into the blotting chamber and the chamber was filled with ice from outside. Proteins were transferred at 50 V for 90 min.

2.4.2.4 Protein detection and visualisation

Protein transfer was checked with Ponceau S red stain (0.5 % Ponceau S in 1 % acetic acid; Sigma-Aldrich, Poole, UK) followed by washing of the Ponceau S twice with TBST Buffer (see Appendix). Membranes were blocked with 5 % skimmed milk powder in TBST Buffer for 30 min at room temperature. Membranes were further incubated for 90 min at room temperature (or at 4° C overnight) with either Monoclonal Anti-α-Tubulin CloneB-5-1-2 (Sigma-Aldrich, Poole, UK) at 1:2000, P-Cofilin antibody (Cell Signalling Technology, Danvers, USA), Cofilin antibody (Cell Signalling Technology, Danvers, USA), Profilin-1 antibody (Cell Signalling Technology, Danvers, USA) or Gelsolin antibody (Abcam, Cambridge, UK) at 1:2000.

After being washed three times for 5 min with TBST buffer, the membranes were incubated with an appropriate secondary horse-radish-conjugated anti-mouse or anti-rabbit antibody (Cell Signalling Technology, Danvers, USA) at 1:2000 for 45 min on a rocker. Membrane(s) were washed three times for 5 min, as previously described.

Pierce®ELC Western Blotting Substrate (Fisher Thermo Scientific, Loughborough, UK) an enhanced chemiluminescence (ECL) reagent was added for 1 min for visualization under red light and then exposed onto film for 30 s (Fuji Medical X-ray film, Bedford, UK). The film was exposed to the membrane for 30 s. The film was then removed and placed in developer (Kodak, Hemel Hempstead, UK) for approximately 3 min before being placed in Fixer (Kodak, Hemel Hempstead, UK) for 5 min. The film images were scanned to obtain a digital image and the amount of protein in samples and was estimated using densitometry analysis. ImageJ®. The intensity of the band emission and background emission of the gel and the background was subtracted from the intensity of each band.

2.5 Data Analysis

2.5.1 Viability analysis

To determine cell viability post impact CLSM data was analysed via Imaris 7.1 (Bitplane, Zurich, Switzerland). *In situ* chondrocytes demonstrate a wide fluorescent range and it was therefore necessary to determine an optimal baseline fluorescence threshold (BT) by using Imaris 7.1 calibrated to an initial volume obtained from cells in isotonic saline with live cells (green spots) and dead cells (red spots; Figure 2.6*). The optimal BT for calcein AM and PI were, 65 % of the maximum intensity and 15 % of the maximum intensity respectively.
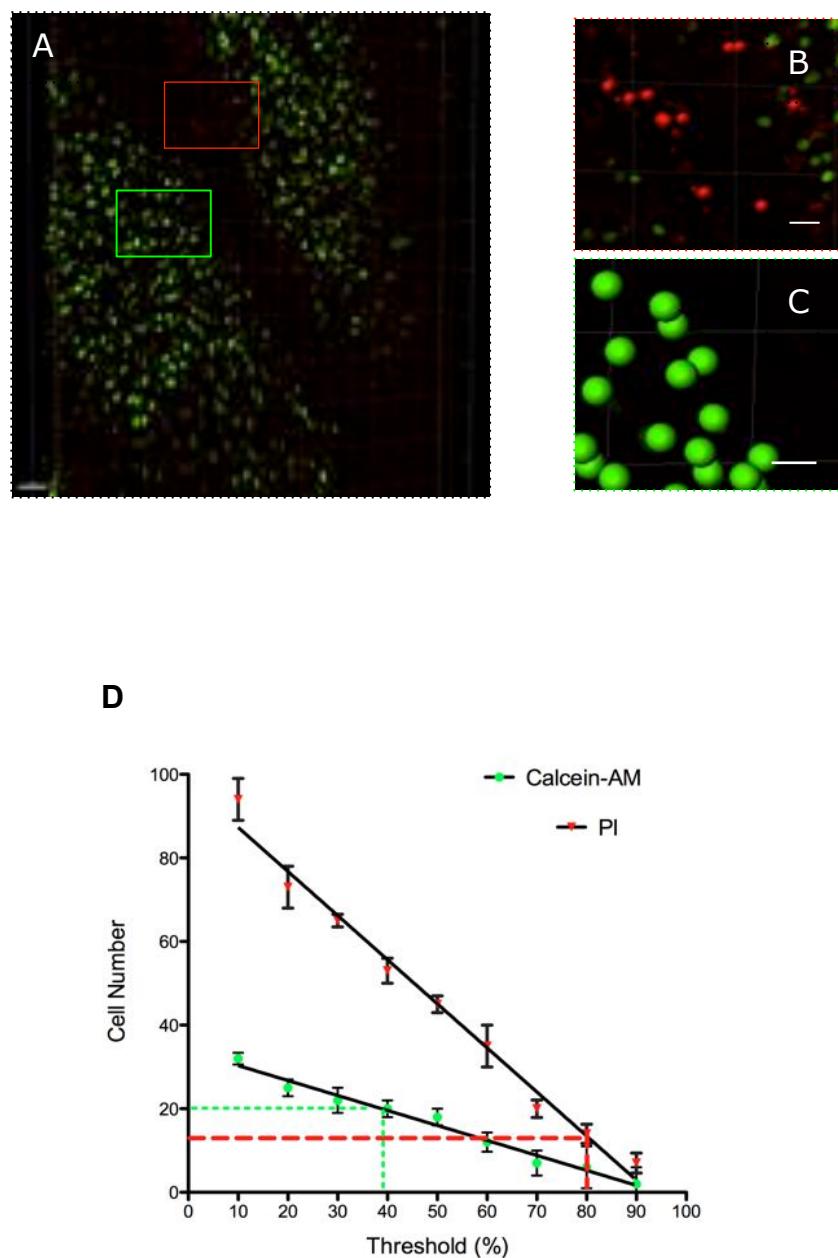
Figure 2.6 'Spot' analysis optimisation.

A) Cartilage explants were stained with 5 μM Calcein-AM and 1 μM PI to visualise live (green) cells and dead (red) cells. Samples were acquired with CLSM. B) Visually countable number (counted by eye) of dead cells with spot analysis applied. C) Visually countable (counted by eye) number of live cells with spot analysis applied. D) Cell number was plotted against threshold percentage and linear regression applied. Actual cell number was used to determine the optimal threshold percentage to be used with spot analysis. (A) Bar =100 μm (B) Bar = 5 μm (C) Bar = 10 μm

2.5.2 Volume analysis

In situ chondrocytes demonstrate a range of fluorescence (Bush *et al.,* 2005) therefore, it was necessary to determine optimal fluorescent baseline threshold (BT). Briefly, the system was calibrated with fluorescent latex beads Fluoresbrite™ (Polyscience Inc., Eppelheim, Germany) of a known volume ($540 \ \mu m^3$), imaged in the same way as bovine cells and 'isosurface' applied with spectrum threshold perentage (10-100 %). A linear relationship was found between the threshold and the apparent volume with correct volume determined at 60 % threshold (Figure 2.7).

Cell volume changes were ascertained by importing the data from Leica SP2 (Leica Microsystems, Milton Keynes, UK) into Imaris 7.1 (Bitplane, Zurich, Switzerland) and application of a feature of the software termed 'Isosurface'. An 'Isosurface' was added to each individual cell and the volume calculated, Initial images were acquired in control media 280 mOsm for cartilage explants and 380 mOsm for isolated chondrocytes.
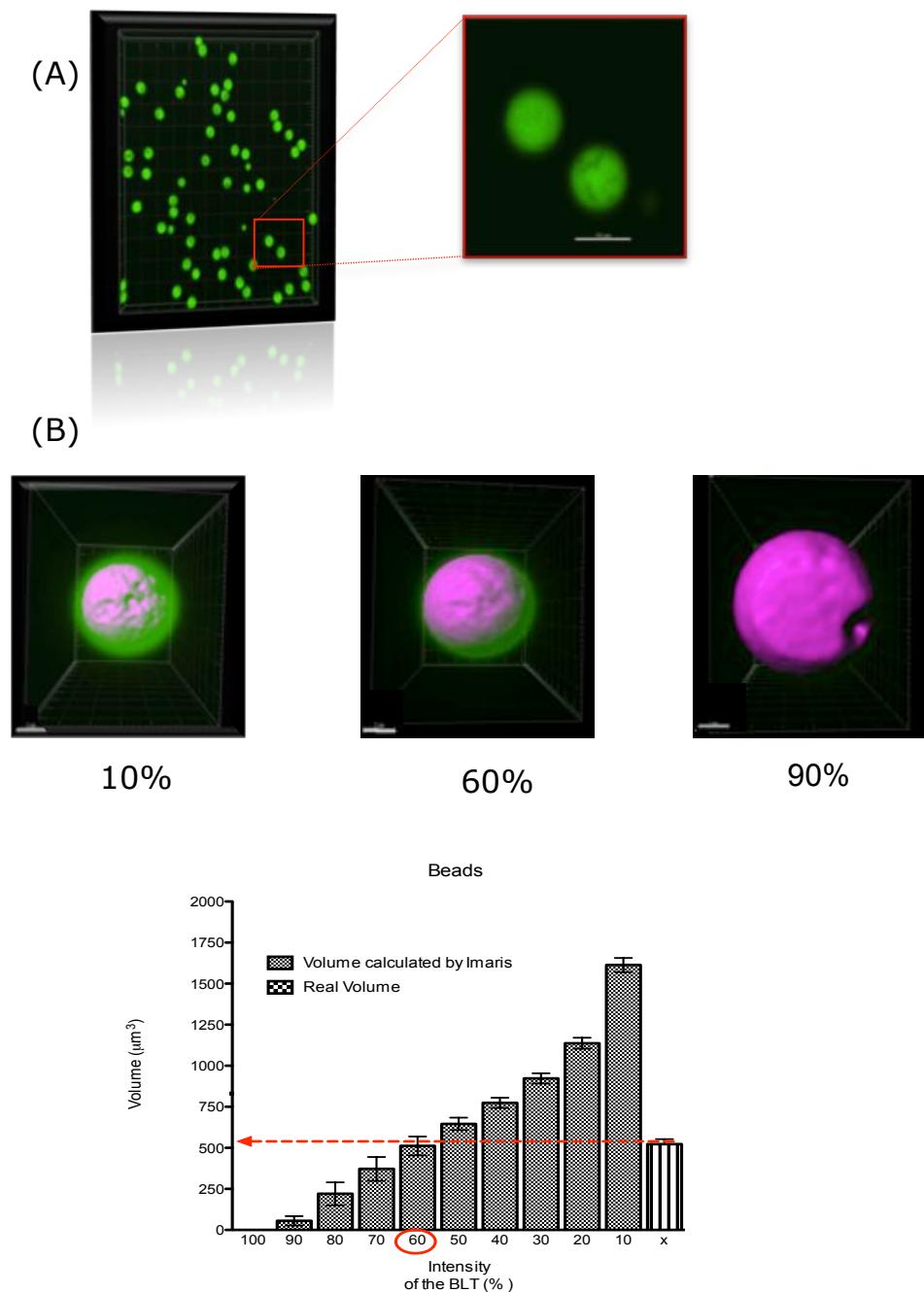
Figure 2.7 Optimization of volume analysis using fluorescent beads with known Volume.

A) *Latex beads overview*

B) *Application of percentage thresholds to a single bead*

Apparent bead volume was used to elucidate the optimal threshold. The percentage threshold was plotted against the calculated volume from Imaris and actual bead volume was used to elucidate the actual threshold percentage.

2.5.3 Actin Analysis

The organization of the actin cytoskeleton was studied using Leica Confocal Software LCS Lite (Leica Microsystems, Heidelberg, Germany) by using linear profiling across the cell diameter (Kerrigan and Hall, 2005). Briefly, a line profile was drown to the centre of each cell perpendicular in the x-y axis and parallel in z-axis (Figure 2.8) and then data were exported to Microsoft$^{®}$ Excel for further analysis.

2.5.4 Analysis of changes in calcium

Changes in intracellular calcium were studied using FLUOstar OPTIMA plate reader (BMG Labtech, Aylesbury, UK). Through the instrument's liquid-handling system osmotic gradient (10-90 %) were applied 55 s after the beginning of each recording and followed for 300 s. The gradient for the calcium influx curve was calculated to determine the variations in the calcium influx in respect to the external osmotic pressure. The gradient of a line in the plane containing the *x* and *y* axes is generally represented by the letter *m*, and is defined as the change in the *y* coordinate divided by the corresponding change in the *x* coordinate, between two distinct points on the line. This is described by the following equation:

$$m = \frac{\Delta y}{\Delta x} = \frac{y_2 - y_1}{x_2 - x_1} = \frac{rise}{run}$$

m=slope
y= y axe *(rise)*
x= x axe (run)

for the purposes of this research the slope was calculated for the Ca^{2+}rise until Ca^{2+} maximum was reached (Figure 2.9).
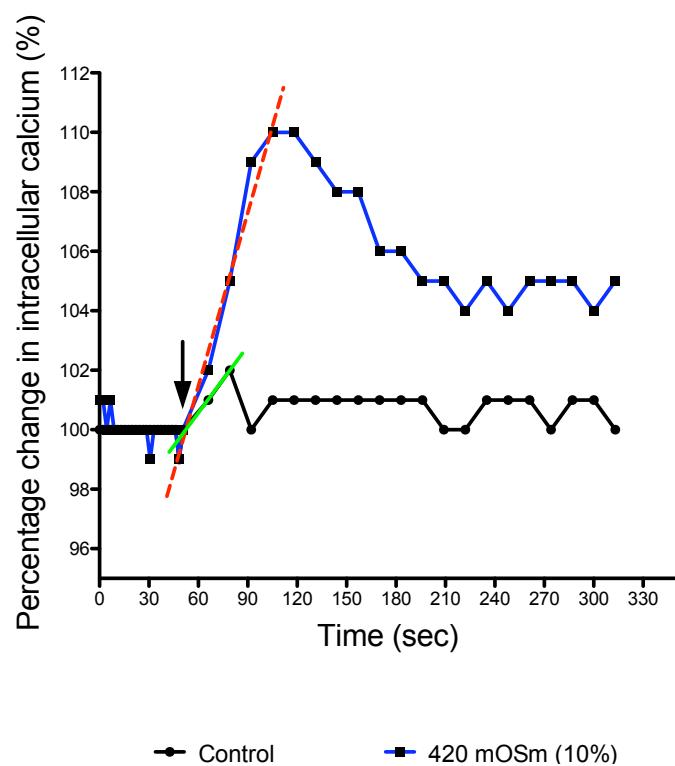
Figure 2.9 Slope analyses to determine calcium influx.

Freshly isolated bovine cells were plated on 12 well plates and stained with 2 μM Fluo-4, AM for 30 min. Calcium changes were studied using FLUOstar OPTIMA plate reader (BMG Labtech, Aylesbury, UK) and the recorded results were analysed with slope analysis. Arrow represents when the increased extracellular osmolarity was applied and the straight line that is tangent to the curved peak represents the function of slope of a curve in the plain.

2.6 Data Presentation

All data were expressed as means ± standard error of the mean (s.e.m.), the number of explants used within an experiment was given with (N) whilst the total number of cells analysed (n) was also given. Graphs were plotted in GraphPad PRISM 5 (GraphPad Software, La Jolla, USA). Changes in cell volume were calculated using Imaris 7.1 (Bitplane, Zurich, Switzerland). All statistical analysis (Student's unpaired t-tests) and significant differences were given as *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ and performed using Microsoft® Excel 2011 for Mac.
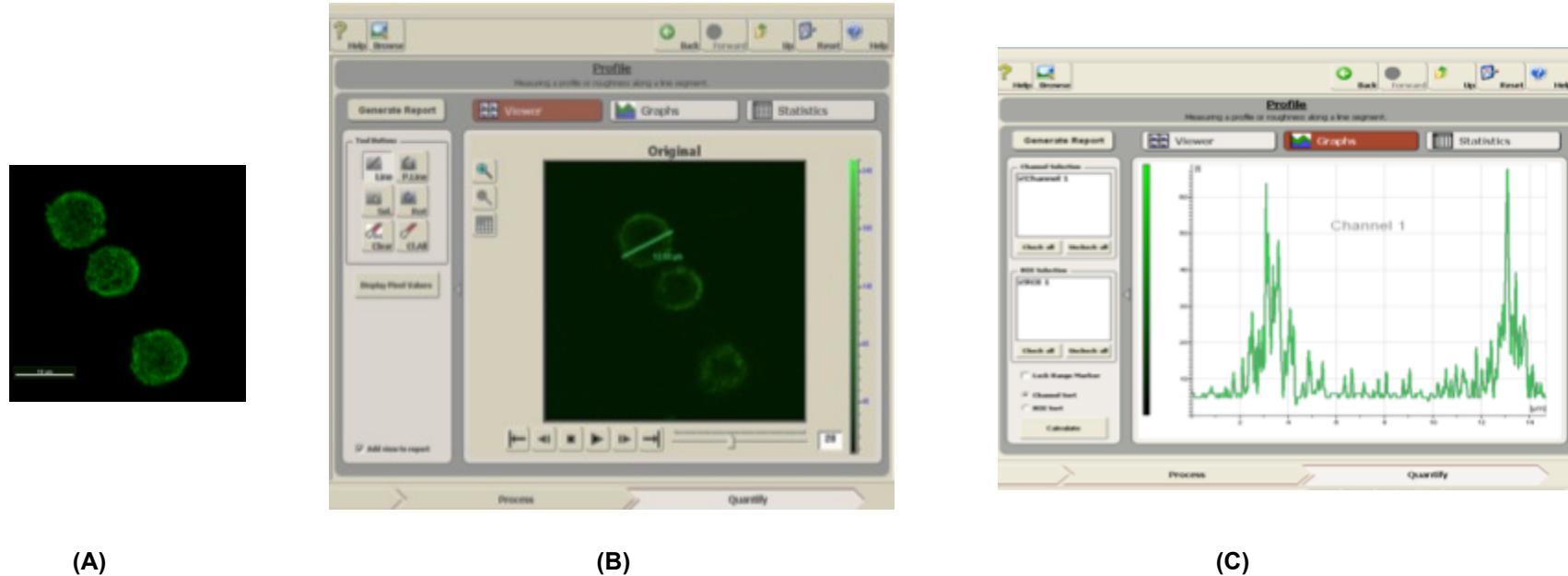
Figure 2.8 Actin Cytoskeleton organization analysed using Liner Profiling Method.

(A) Overview image taken by CLSM. Bovine cells were fixed in 4 % para-formaldehyde and stained with Alexa 488-phalloidin. (B) Actin cytoskeleton was studied using Leica Confocal Software LCS Lite, whereby lines were drawn horizontally and vertically across the diameter of each individual cell and the fluorescence along the line recorded. (C) Fluorescence profile of a cell recorded along the line. Software LCS Lite, whereby lines were drawn horizontally and vertically across the diameter of each individual cell and the fluorescence along the line recorded. (C) Fluorescence profile of a cell recorded along the line.

CHAPTER 3
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3.   **EXPERIMENTAL CHAPTER: Osmotic Sensitivity of Chondrocytes and Chondro-protection in Response to Mechanical Trauma.**

3.1 Introduction

Whilst mechanical loading is essential for the synthesis and regulation of a viable extracellular matrix (ECM), excessive mechanical loading results in cartilage damage and formation of tissue lesions or fissures, comprising the integrity of the tissue (Verteramo & Seedhom, 2007). Excessive mechanical loading on cartilage can be modelled using a drop-tower whereby a pre-defined mass is dropped onto a piece of cartilage from a defined height and simulates focal impact forces (Aspden *et al.,* 2002). Chondrocytes can be protected from the mechanical trauma elicited following drop-tower impact by pre-incubating the tissue in hypertonic media, although the exact mechanism of protection is unclear (Bush *et al*., 2005), an increase in extracellular osmolarity leads to a decrease in chondrocyte cell volume. When working with chondrocytes it is important to note that they behave as perfect osmometers when exposed to a hypertonic environment and thus variation in volume can have significant effect on their biology and matrix metabolism also known to be influenced by cell volume (Urban, 1994, Bush and Hall, 2005, Bush *et al.,* 2005).

Mechanical cartilage trauma is a major concern in high-impact load-bearing sport (Kujala *et al.,* 1995, Lequesne, 1997, Saxon, 1999, Roos, 2005, Urquhart *et al.,* 2007). Therefore, the elucidation of cartilage damage as a consequence of mechanical trauma is of paramount importance for both the casual, professional and elite sports person. Chondrocyte death has a major impact on the degeneration of articular cartilage depending on strain rate and mechanical load (Chen *et al.,* 2001, Bush *et al.,* 2005). Recent studies have proposed that chondrocyte viability is intrinsically linked to matrix integrity and high levels of cartilage injury results in increased cell death; necrosis and apoptosis (Chen *et al.,* 2001, Goldring and Goldring, 2010). In cartilage, it has been shown that following a blunt impact there is a 'wave of chondrocyte death' that progresses further from the impact area (Bush *et al.,* 2005).

Previous studies evaluating impact damage using mechanical models, show that one of the key effects on morphology are alterations in cell volume, whereby decreasing cell volume leads to a reduction in cell death, thus making

them less susceptible to mechanical trauma (Bush *et al.,* 2005, Amin *et al.,* 2009). Conversely, swelling of *in situ* chondrocytes by reduced extracellular osmolarity, elevates their sensitivity and increases their cell death to mechanical loading (Bush and Hall, 2003, Lotz *et al.,* 2004, Bush *et al.*, 2005, Grogan and D'Lima, 2010).

3.1.1 Chapter aims

This study aims to expand on the previous work described by investigating:

1) Changes in chondrocyte viability and volume in response to mechanical trauma;
2) Identification of the optimal osmotic challenge for chondro-protection;
3) The role of extracellular osmolarity in chondro-protection;
4) The role of actin cytoskeleton in chondro-protection;
5) To determine the influence of extracellular calcium on chondrocyte death.

3.2 Results

3.2.1 Osmotic Sensitivity and Cell Viability in Response to Mechanical Trauma

The effects of osmotic sensitivity on cell viability were determined initially by investigating cellular response to a gradient of extracellular osmotic challenges. Articular cartilage explants were removed from bovine metacarpal/metatarsal phalangeal joints and incubated at 37° C with DMEM of 280 mOsm (control), 380 mOsm, 420 mOsm or 540 mOsm for 1 h, followed by a single impact (see section 2.2) and analysed by CLSM for cell volume and cell viability.

Single mechanical impact caused chondrocyte cell death and injury at discrete areas on the cartilage explants (Figure 3.1). Step-wise increases in extracellular medium osmolarity were utilised to determine the optimal osmotic challenge affording chondro-protection within *in situ* bovine cartilage explants. Cartilage explants incubated in 280 mOsm and not subjected to impact showed no significant changes in cell viability over the time period thus validating the experimental method. Conversely, impacted control samples (280 mOsm), were observed to show significant decrease in cell viability (89.69 ± 0.89 %) at 30 min post impact ($p<0.05$). Interestingly, following an increase in extracellular osmolarity, a challenge of 280 to 380 mOsm resulted in a 30 % ($p<0.001$) reduction in cell death and a challenge of 280 to 420 mOsm further reduced impact-mediated cell death by 50 % ($p<0.01$) at 30 min post impact (Figure 3.2). At 540 mOsm it was noted that present death cell (PDC) was decreased in comparison to the control samples but it was not noted to be significantly different 30 min post impact ($p>0.05$) in comparison to control samples with a value of 92.07 ± 1.54 % viable cells.

Having determined the effects on different osmotic loads on chondrocyte viability (Figure 3.2), the changes on cell volume was subsequently determined. Volume in control chondrocytes (i.e. not impacted, 280 mOsm) of *in situ* chondrocytes was 716.14 ± 36.68 μm^3 and remained constant throughout the 30 min period. These data compared favourably to previous published data

(Bush *et al.,* 2005, Parker *et al.,* 2010). Cell volume post-impact did not change significantly in the control samples, which only decreased by 3 % at 30 min post impact (694.18 ± 25.8 μm^3, p>0.05). An increase in extracellular osmolarity to 380 mOsm, 420 mOsm & 520 mOsm led to a significant decrease in chondrocyte volume prior to impact (Figure 3.3A), with cell volumes decreased by 20 % (578.46 ± 26.85 μm^3; p<0.01), 29 % (505.90 ± 21.90 μm^3; p<0.001) and 39 % (440.13 ± 16.42 µm3; p<0.001) respectively being observed (Figure 3.3B). Following post mechanical impact no significant changes in cell volume were observed 30 min post impact with 561.39 ± 25.55 μm^3 (380 mOsm), 493.12 ± 21.90 μm^3 (420 mOsm), 445 ± 18.90 μm^3 (540 mOsm) (p>0.05) being determined. These data therefore suggest that within the 30 min post impact cells have not been significantly altered by the single impact and the initial shrinking of the cell volume has protected the cells from the effect of mechanical impact.
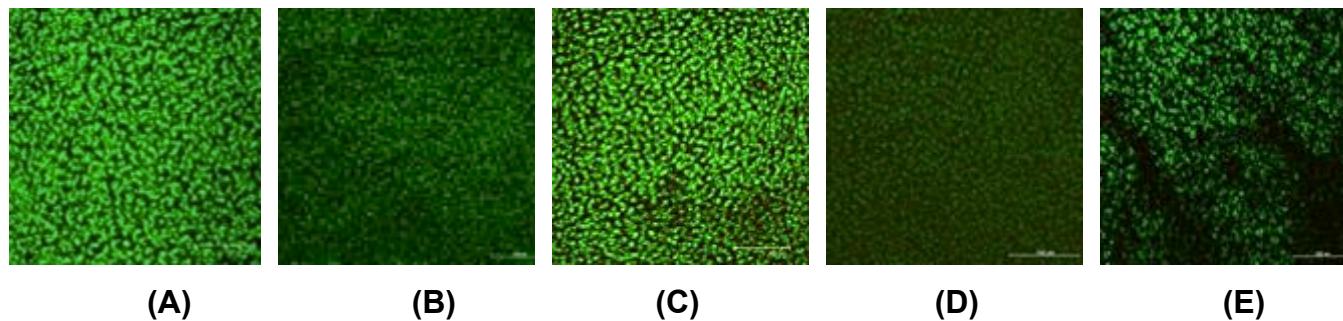
Figure 3.1 Effect of alterations in osmolarity on chondrocyte viability following single mechanical load.

Explants were pre-treated with control isotonic conditions or hypertonic DMEM 380, 420 and 540 mOsm for 1 h and subject to a single mechanical load. Samples were loaded with Calcein-AM (5 µM) and PI (1 µM) and images were taken at 30 min post 10 cm impact. (A) non impacted cartilage explants in 280 mOsm (B) control sample 30 min post impact (C) sample in hypertonic DMEM 380 mOsm 30 min post impact (D) sample in hypertonic DMEM 420 mOsm 30 min post impact (E) sample in hypertonic DMEM 540 mOsm 30 min post impact. Bar = 100 µm. Images for all panel (A-E) were acquired by axial confocal laser scanning microscopy (CLSM) projections imaging the superficial zone of cartilage 'top-down' from the articular surface.
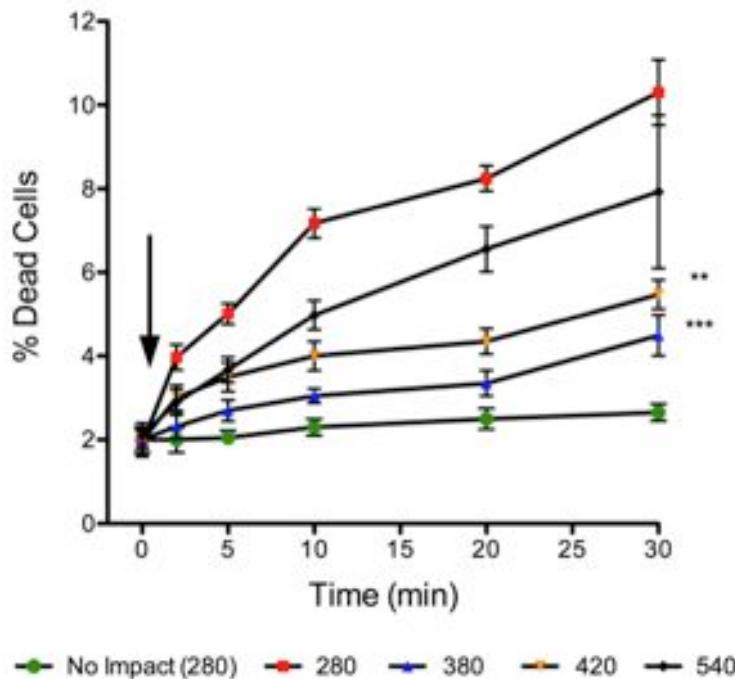
Figure 3.2 Chondro-protective role of hyperosmotic pre-treatment on cell viability following single mechanical load.

Explants were pre-treated with control isotonic conditions or hypertonic DMEM 380, 420 and 540 mOsm for 1 h and subject to a single mechanical load. Samples were loaded with Calcein-AM (5 µM) and PI (1 µM) and images were taken at 2, 5, 10, 20 & 30 min post impact. Data are mean +/- SEM of N=9, *p<0.05, **p<0.01, ***p<0.001 all vs. control (280 mOsm).

Osmolarity (mOsm)	Viability at 2 min (%)	Viability at 30 min (%)
No Impact 280	98.00 ± 0.30	97.35 ± 0.20
Control (280)	96.03 ± 0.30	89.69 ± 0.89
380	97.68 ± 0.32*	95.50 ± 0.54***
420	97.03 ± 0.29*	94.39 ± 0.34 **
540	97.10 ± 0.30	92.07 ± 1.54

Table 3.1 Role of hyperosmotic pre-treatment on cell viability following single mechanical load.

A

| CONTROL | HYPER 380 mOsm | HYPER 420 mOsm | HYPER 540 mOsm |

(B)

(C)
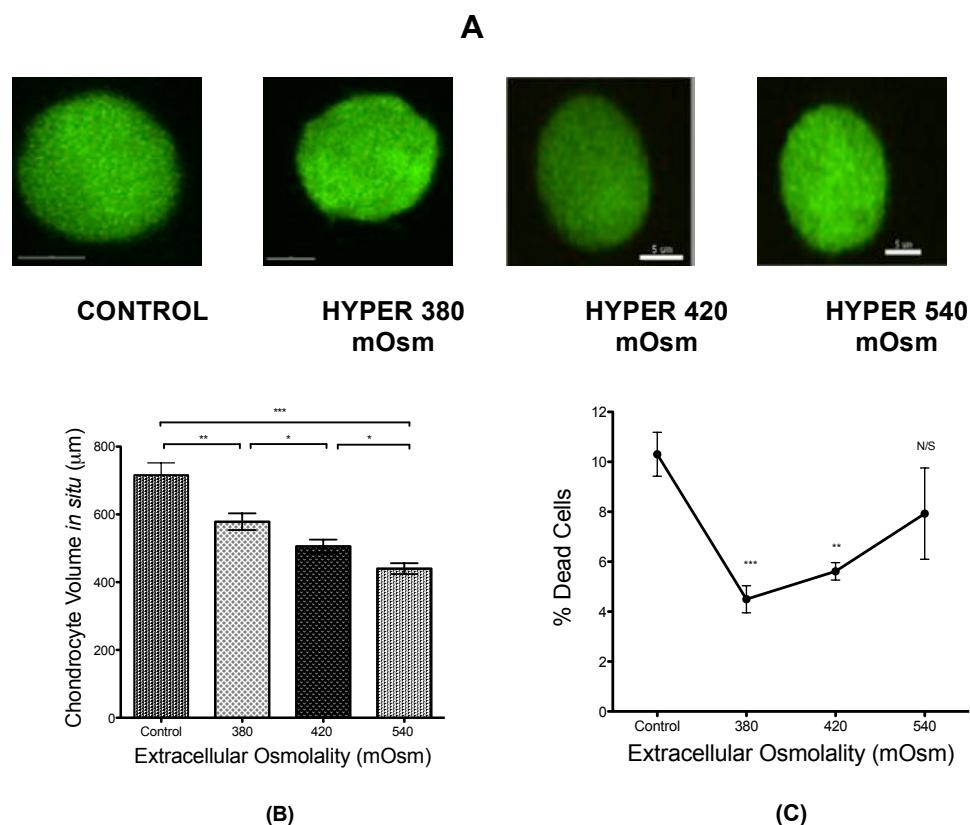
Figure 3.3 Hyperosmotic pre-treatment decreases chondrocyte cell volume post mechanical trauma.

Cartilage explants were pre-treated for 1 h with control isotonic or hypertonic DMEM of 380, 420 and 540 mOsm that represented 30 %, 50 % and 90 % increase of extracellular osmolarity respectively and subject to a single mechanical load. Samples visualised by CLSM were loaded with Calcein-AM (5 μM) and PI (1 μM) and images were taken 30 min post impact (A). Isosurface volume analysis were used to quantify cell volume and to quantify cell viability, spot analysis method was employed. Data are mean +/- SEM of N=9 n=60 from 3 distinct experiments (B) and N=9 from 3 distinct experiments (C). *p<0.05, **p<0.01, ***p<0.001 all vs. control (280 mOsm).

Osmolarity (mOsm)	Chondrocyte volume (μm^3)	Viability (%)
Control 280	716.14 ± 36.68	89.69 ± 0.89
380	578.46 ± 26.85 *	95.50 ± 0.54***
420	505.90 ± 21.90 **	94.39 ± 0.34 **
540	440.13 ± 16.42 ***	92.07 ± 1.54

Table 3.2 The effect of hyperosmotic pre-treatment on chondrocyte cell volume and viability post mechanical trauma.

3.2.2 Effects of medium osmolarity on calcium signalling

The biophysical mechanisms of mechanical signal transduction are not fully understood but are believed to involve changes in osmolarity (Pritchard *et al.,* 2002). The chondrocyte plasma cell membrane is relatively permeable and increasing extracellular osmolarity leads to decrease of cell volume (Kerrigan *et al.,* 2006). Alteration of cell volume is responsible for triggering volume recovery mechanisms that involve activation of ion channels and intracellular signalling cascades in an effort to restore cell volume (O'Neil, 1999, Amin *et al.,* 2008). In particular, intracellular calcium $[Ca^{2+}]_i$ represents an ubiquitous signalling mechanism and is involved in processes as diverse as cell division and apoptosis (Berrige *et al.,* 1998, Roberts *et al.,* 2001). Cells utilise two sources of Ca^{2+} for the generation of signals: Ca^{2+} released from the intracellular stores and activation of plasma membrane ion channels resulting in Ca^{2+} entry (Wilkins *et al.,* 2000). Ca^{2+} signals can be modulated in space and time and this influences $[Ca^{2+}]_i$ concentration, thereby suggesting Ca^{2+} signalling plays an important role in regulatory volume control and is a mediator in mechanotransdution (Amin *et al.,* 2008, Kerrigan and Hall, 2008).

The series of experiments demonstrated that replacement of the control solution with identical solution resulted in no significant changes of $[Ca^{2+}]_i$ however, replacement of the isotonic solution with hyperosmotic solution caused an increase spike of $[Ca^{2+}]_i$. Therefore, it was important to determine the behaviour of $[Ca^{2+}]_i$ in relation to different osmotic gradients in order to understand the level of protection offered by increased osmolarity. To closely monitor the volume regulatory behaviour a number of hyperosmotic challenges were used, where medium osmolarity was altered by 10, 20, 30, 40, 50, 60 & 90 %. From the raw data recorded a significant increase of calcium approximately a 10 % was observed in response to 10% osmotic change, 20 % and 30 % increased osmolarity (Figure 3.6) compared to only approximately 5 % or less for 40%, 50%, 60%, and 90% hyperosmotic challenges (Figure 3.4).

Having determined the percentage increase in intracellular calcium, the rate of the changes was then calculated. The gradient analysis (see Materials and Methods, Section 2.5.4) was used to allow for a better investigation and understanding of the effect of increased osmolarity on freshly isolated cells. Chondrocytes perfused with low hypertonic challenges (420 mOsm & 460 mOsm), exhibited increase in Ca^{2+} by 0.58 ± 0.01 and 0.57 ± 0.03 relatively (Figure 3.5), acute hypertonic challenges above 30 % (540 mOsm, 570 mOsm, 720 mOsm) did not significantly increase rate in Ca^{2+} thus showing that calcium changes are not respectively related to cell shrinkage and suggesting that additional mechanisms are involved in volume regulatory changes.
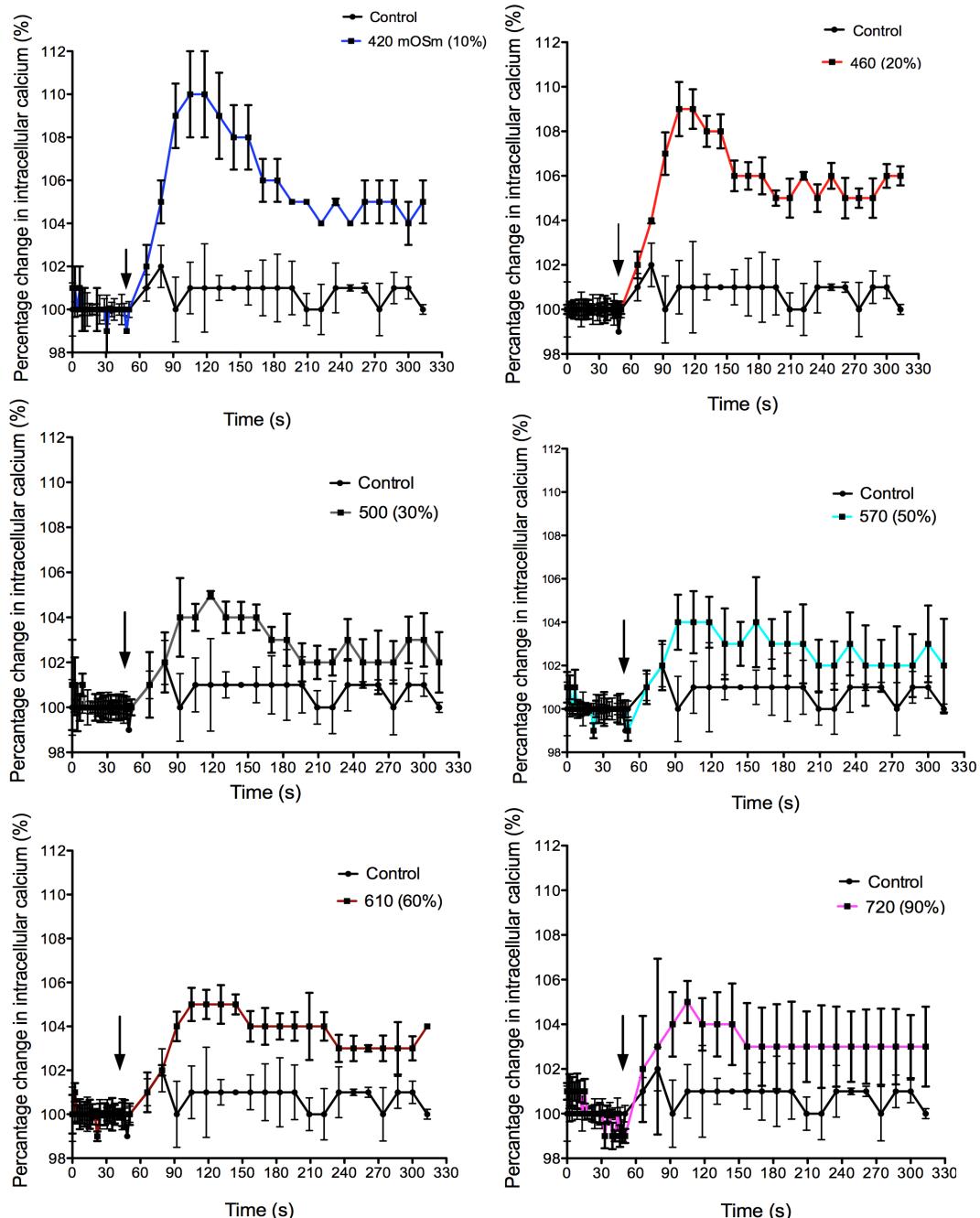
Figure 3.4 Representative traces of extracellular calcium changes in response to hyperosmotic stress in freshly isolated chondrocytes (Po).

Freshly isolated bovine articular chondrocytes in 380 mOsm control saline were incubated with fluo-4 AM (2 μM, 30 min, 37° C) for the measurement of $[Ca^{2+}]_i$. The initial resting intensity was recorded for 55 s in an isotonic saline (380 mOsm) and then a hyper osmotic challenge (420 mOsm; 10 %, 460 mOsm; 20%, 500 mOsm; 30 %, 570 mOsm; 50 %, 610 mOsm; 60 %, 720 mOsm; 90 %) was applied shown by arrow. For intracellular Ca^{2+} measurements, fluorescence emissions at 516 nm was measured and data is shown in percentage of n=6 samples from 3 distinct experiments.
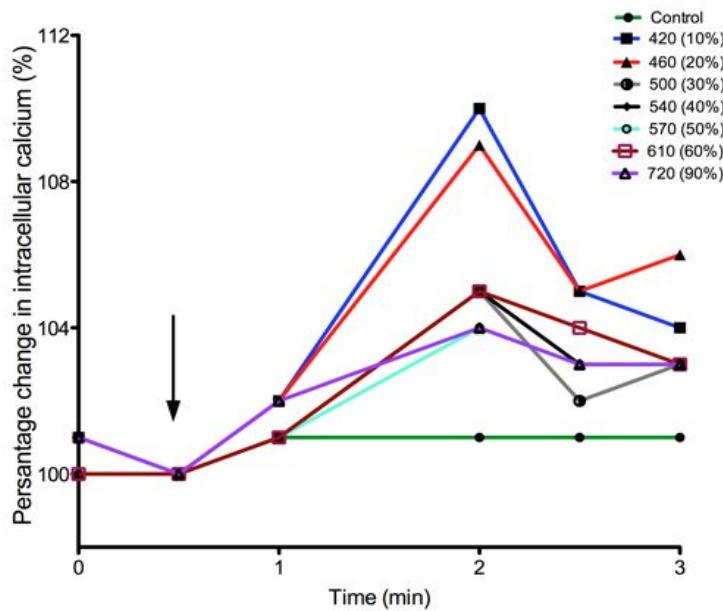
Osmolarity Gradient change (%)	Initial rate of change m=rise/run (fluorescence/time^{-s})
Control	0.15 ± 0.01
10	0.58 ± 0.01 *
20	0.57 ± 0.03 *
30	0.33 ± 0.06
40	0.38 ± 0.08
50	0.18 ± 0.01
60	0.20 ± 0.01
90	0.40 ± 0.01

Figure 3.5 Changes in Ca^{2+} following addition of hyper osmotic gradient.

*Freshly isolated bovine cells were plated on 12 well plates and stained with 2 μM Fluo-4, AM for 30 min. Calcium changes were studied using FLUOstar OPTIMA plate reader (BMG Labtech, Aylesbury, UK) and the recorded results were analysed with slope analysis. Panel (A) Arrow represents when the increased extracellular osmolarity was applied. Max rise ration determined by slope analysis. Data are mean +/- SEM of n=6 samples *p<0.05, **p<0.01, ***p<0.001 all vs. control.*

3.2.3 Extracellular calcium and its role in chondrocyte death in response to Mechanical Trauma

Changes in calcium have been implicated in cell volume regulation (Erickson *et al.,* 2003, Kerrigan and Hall, 2008, Amin *et al.,* 2008, Pritchard *et al.,* 2008) and in chondrocyte death associated with mechanical injury (Amin *et al.,* 2009, Huser and Davies, 2007) and importantly play a role in many aspects of mechanotransduction (Kerrigan *et al.,* 2006, Amin *et al.,* 2009, Han *et al.,* 2012). Here the effect of extracellular calcium and any potential calcium influx on chondrocyte death following mechanical injury was determined in bovine explants incubated with EGTA (2 mM), thus removing extracellular calcium (Kerrigan and Hall, 2005).

Bovine cartilage explants were incubated in control DMEM (280 mOsm), EGTA (2 mM; 280 mOsm) and EGTA (2 mM) with increased osmolarity 380 mOsm and subjected to impact by drop tower. Post mechanical trauma, cell death was not significantly altered in samples with 10.34 ± 0.77 % (control), 10.95 ± 0.62 % (EGTA) and 9.73 ± 0.62 % (EGTA 380 mOsm) PDC respectively over 30 min (Figure 3.6A).

Having determined the changes in chondrocyte viability following impact changes in cell volume were then investigated. Significant changes in cell volume were observed in *in situ* chondrocytes, whereby the cell volume of chondrocytes recorded in the presence of 280 mOsm EGTA decreased from 716.14 ± 36.68 μm^3 in control samples to 600.32 ± 18.33 μm^3 ($p<0.05$) and 486.40 ± 20.33 μm^3 ($p<0.001$) for *in situ* chondrocytes incubated in 380 mOsm EGTA. Data suggesting that the extracellular calcium might play a role in the later stages post-mechanical injury but not within the first 30 min (Figure 3.6B). These data also suggests that shrinkage and the presence of calcium are both important factors in chondro-protection.

(A)

(B)
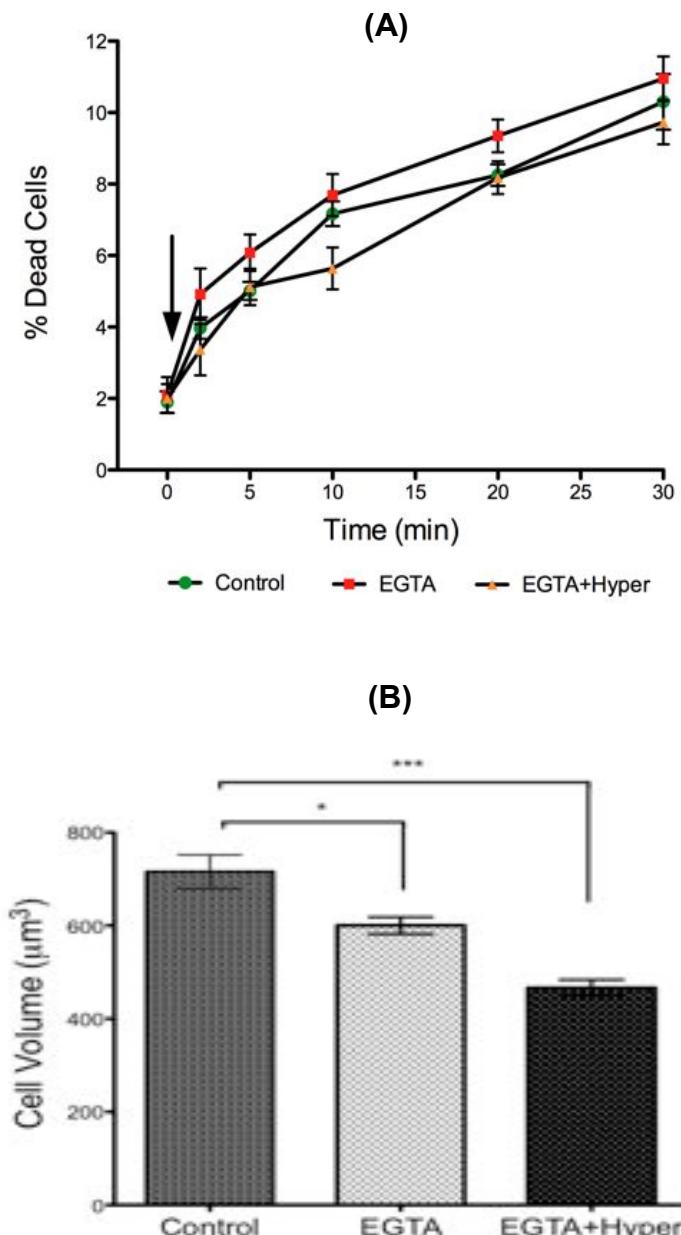
Figure 3.6 Short-term chondrocyte response to mechanical trauma and the role of calcium influx.

Bovine cartilage explants were pre-treated with control isotonic DMEM, EGTA supplemented (2 mM) and hypertonic (380 mOsm) EGTA (2 mM) supplemented BPS for 1 h and subject to single mechanical load. (A) Samples were loaded with Calcein-AM (5 µM) and PI (1 µM) and images were taken at 2, 5, 10, 20 & 30 min post impact with CLSM. To quantify cell viability Imaris Spot analysis was used N=12 from 3 distinct experiments. Arrow denotes impact point. (B) Samples were stained with Calcein-AM (5 µM) and analysed at 30 min with Imaris Isosurface to quantify cell volume. Data are mean +/- SEM of N=9 and n=50 from 3 distinct experiments. *p<0.05, **p<0.01, ***p<0.001 all vs. control.

As the previous data (see section 3.2.2) suggested role for a calcium influx in the mechanism of cell death, the effect on cell shrinkage on chondro-protection and the role of stretch activated calcium channel (SACC) was then determined. Non-impacted cartilage pre-incubated with 100 μM GdCl$_3$ was evaluated for chondrocyte viability. No differences were observed between treated and un-treated non-impacted explants therefore providing confidence that any changes in chondrocyte death post impact were related to the effect of GdCl$_3$ on SACC. GdCl$_3$ treated cartilage explants were subjected to 10 cm impact and cell viability determined, in control (280 mOsm) DMEM, 3.97 ± 0.30 % of cells were non-viable at 2 min post impact and cell death was increased to 10.31 ± 0.89 % at 30 min. The addition of hypertonic challenge, reduced the initial cell death to 2.18 ± 0.18 % ($p > 0.05$) compared to control samples. Conversely, at 5, 10, 20 & 30 min post impact PDC for cells incubated in hypertonic soluion was observed to be significantly different from the control sample with PDC recorded to be 3.97 ± 0.30 %, measured at 30 min post-impact with 6% decrease in cell viability ($p < 0.001$; Figure 3.7). In contrast, incubation with GdCl$_3$ reversed this protective effect causing a significant increase in cell death such that at 2 min, 10.67 ± 1.12 % ($p < 0.001$) cells were dead which increased to 20.45 ± 3.88 % non-viable cells at 30 min post-impact ($p < 0.01$).
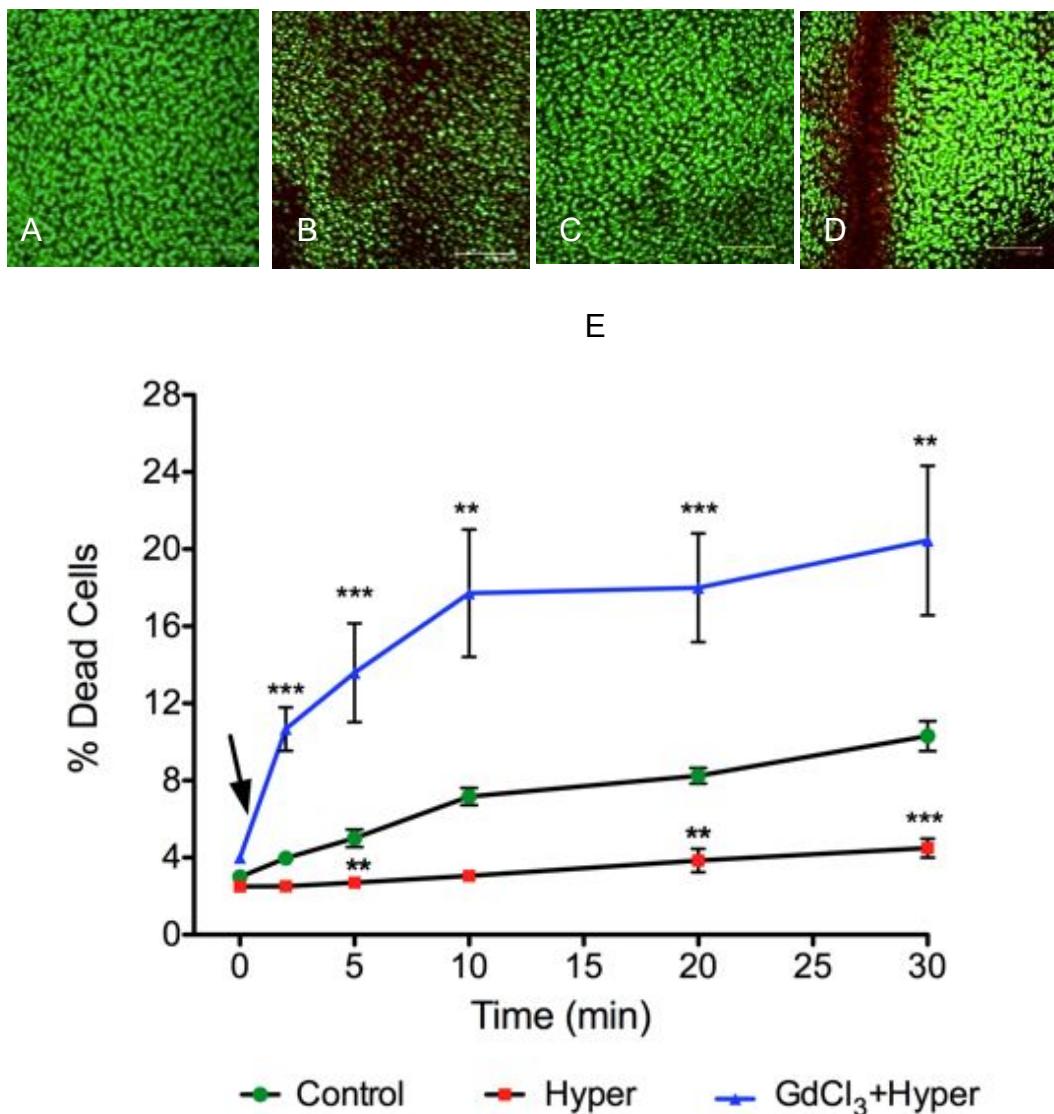
Figure 3.7 The effect of cell shrinkage and gadolinium chloride on chondrocyte viability following single impact.

Cartilage explants were incubated in control and hypertonic DMEM (380 mOsm) with and without the presence of 100 μM GdCl₃. Explants were then subjected to a 10 cm impact and viability determined by Imaris spot analysis. Samples visualised by axial confocal laser scanning microscopy (CLSM) projections imaging the superficial zone of cartilage 'top-down' from the articular surface were loaded with Calcein-AM (5 μM) and PI (1 μM). Images: (A) not impacted sample (B) Control taken after 30 min post impact (C) hypertonic DMEM taken after 30 min post impact (D) hypertonic DMEM with presence of GdCl₃ taken after 30 min post impact. (E) A pre-incubation in a hypertonic DMEM protected chondrocytes from impact trauma by significantly preventing cell death. Conversely, the addition of GdCl₃ reversed and increased cell death following impact. Data are mean +/- SEM of N=9 from three distinct experiment *p<0.05, **p<0.01, ***p<0.001 all vs. control. Arrow denotes impact point. Bar = 300 μm.

3.2.4 The role of F-actin cytoskeleton on chondrocyte death in response to Mechanical Trauma.

Previous studies have shown that remodelling the actin cytoskeleton plays a vital role in maintaining cell structure and integrity of bovine articular chondrocytes as well as being an important step of the cellular response to osmotic stress (Gulilak *et al.,* 1995, Erickson *et al.,* 2003, Blain, 2009). Of relevance it has also been shown that changes in calcium can trigger changes in F-actin and that F-actin may serve as store of Ca^{2+} (Pritchard *et al.,* 2002). In addition, filamentous actin organisation has been noted to be less defined in OA suggesting a defective function of F-actin in OA (Blain, 2009).

To investigate this, bovine explants were incubated for 1 h with Lantranculin B (10 μM), an inhibitor of actin polymerisation (Kerrigan and Hall, 2005). Latrunculin B caused a significant increase in cell death at 30 min post mechanical trauma with 15.0 $\pm$ 0.78 %, (5 %; p>0.001) cell death being observed, no significant differences were observed within the first 2-20 min post impact (2-20 min; p<0.05). In contrast, when Jasplakinolide (5 μM) a stabilizer of F-actin (Bubb *et al.,* 2000) was added, cell viability was increased with only a modest percentage of dead cells being observed at the initial stages post mechanical injury (10 min) of 3.35 $\pm$ 0.30 % (p<0.01) and at 30 min post impact a cell death value of 4.84 $\pm$ 0.46 % (p<0.001; Figure 3.8). Thus confirming that F-actin cytoskeleton plays an important role in the chondro-protective mechanisms. Interestingly, PDC data recorded for cartilage explants incubated in Jasplakinolide was very similar to data recorded for cells incubated in hyperosmotic (380/420 mOsm) DMEM post impact hence implicating the role of F-actin cytoskeleton.
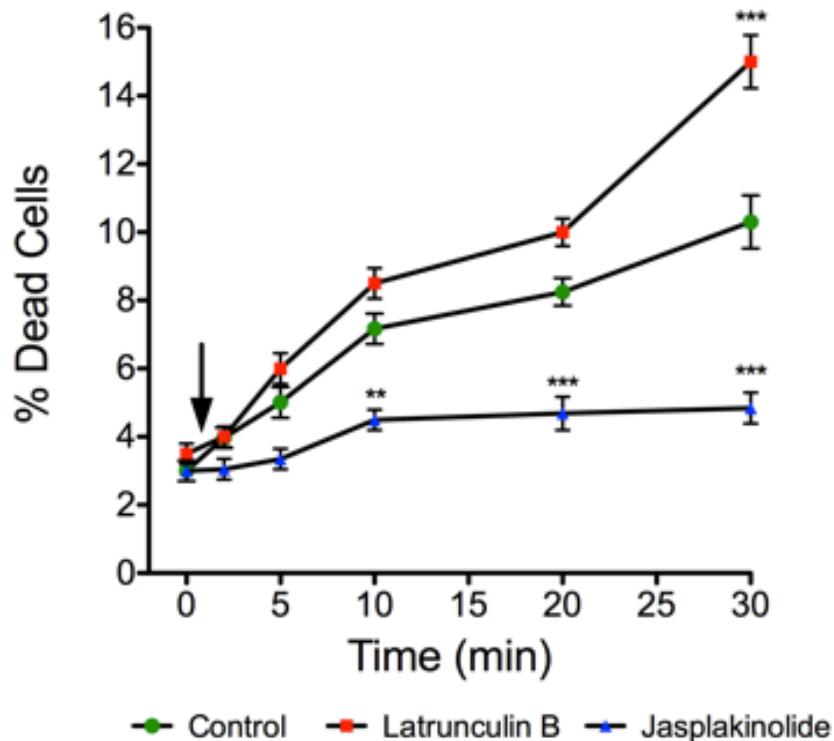
Figure 3.8 The effect of F-actin opposing agents Latrunculin B and Jasplakinolide on chondrocyte viability following single impact.

Cartilage explants were incubated in control isotonic DMEM (280 mOsm), Latrunculin B (10 μM) or Jasplakinolide (5 μM) supplemented DMEM and then subjected to 10 cm impact. Images were taken by CLSM at 2, 5, 10, 20 & 30 min post impact and cell viability quantified via Imaris spot analysis. Data are mean +/- SEM of N=9 from 3 distinct experiments. Arrow denotes impact point. *p<0.05, **p<0.01, ***p<0.001 all vs. control.

3.2.5 Organisation of the polymerised actin cytoskeleton and their response to hyperosmotic and calcium changes within *in situ* and freshly isolated chondrocytes (Po)

The effect of the actin cytoskeleton has been implicated to play a vital role in maintaining cell structure and integrity in different studies (Gulilak *et al.,* 1995, Erickson *et al.,* 2003, Trickey *et al.,* 2004, Pritchard *et al.*, 2008, Blain, 2009) within the previous section it was shown that F-actin re-modelling agents can offer or remove protection in regards to single impact loads. The role of F-actin cytoskeleton and the effect of the hypertonic and calcium changes were then investigated using linear profiling (see Materials & Methods, section 2.5.3). In both *in situ* and freshly isolated bovine chondrocytes F-actin appeared punctate, within *in situ* chondrocytes, it was presented in all zones, however at significantly higher concentrations within the SZ (65.43 ± 3.49 AU; $p<0.01$) compared to the MZ and DZ. No significant differences were found between the MZ 56.49 ± 6.36 AU and DZ 53.10 ± 0.21 AU ($p>0.05$; Figure 3.9)

An increase in extracellular osmolarity (280-380 mOsm) resulted in a significant increase in cortical actin concentration ($p<0.05$) for the SZ (84.79 ± 2.36 AU) but remained unchanged for both the MZ and DZ with 60.81 ± 7.85 AU and 52.80 ± 3.45 AU respectively, in the relative concentration of cortical actin (Figure 3.10). Following a 1 h pre-incubation with $GdCl_3$ there was a significant ($p<0.01$) inhibition of actin polymerisation in the SZ (44.89 ± 0.85 AU) and interestingly a 30 % decrease in cortical actin labelling for both MZ and DZ ($p<0.05/p<0.05$). Interestingly, pre-incubation with 2 mM EGTA (chelator for Ca^{2+}) prevented the hypertonic induced F-actin rise for the SZ cells (70.75 ± 2.36 AU ($p<0.01$)). Whilst in the MZ and DZ there was a reduction in the overall concentration of cortical actin to 44.01 ± 4.69 AU ($p<0.01$) with a more profound effect in the DZ 37.41 ± 1.38 AU ($p<0.05$).

(A)

(B)
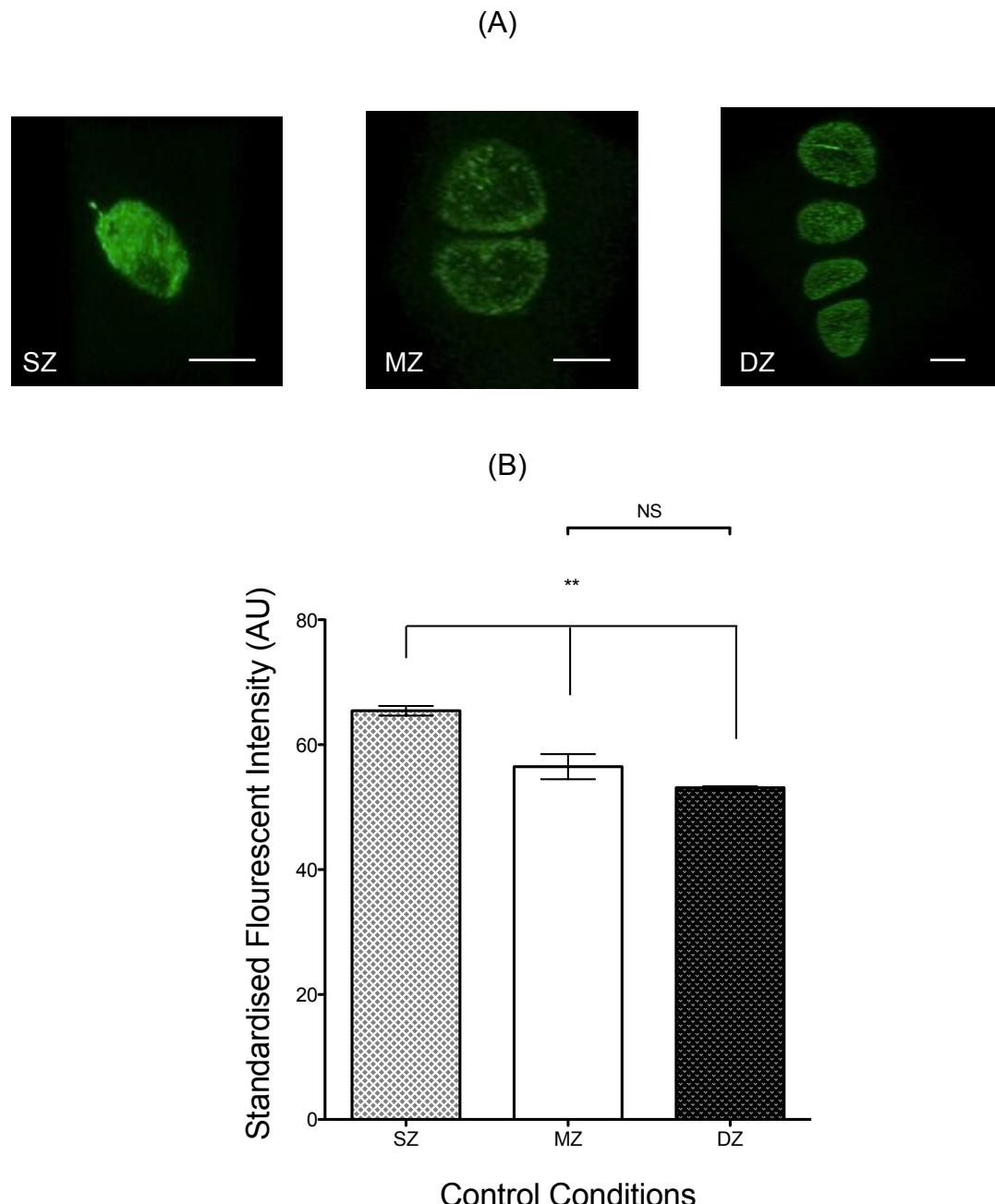
Figure 3.9 Actin organisation within *in situ* bovine articular cartilage as determined by Confocal Laser Scanning Microscopy.

To determine the role of the actin cytoskeleton in chondro-protection cartilage explants were incubated in control 280 mOsm DMEM, fixed and the polymerised actin cytoskeleton labelled with Alexa 488-phalloidin. Images were acquired by coronal CLSM projection of cartilage and deconvolved (by specialist software). Panel (A) shows the discrepancies within the cell shape of chondrocytes. Images were aquired transversely to the articular surface. Bar = 5 µm Panel (B) Shows the increase in polymerisation of the actin cytoskeleton in the SZ. Data are mean +/- SEM of N=9, n=30 *p<0.05, **p<0.01, ***p<0.001 all vs. SZ cells.
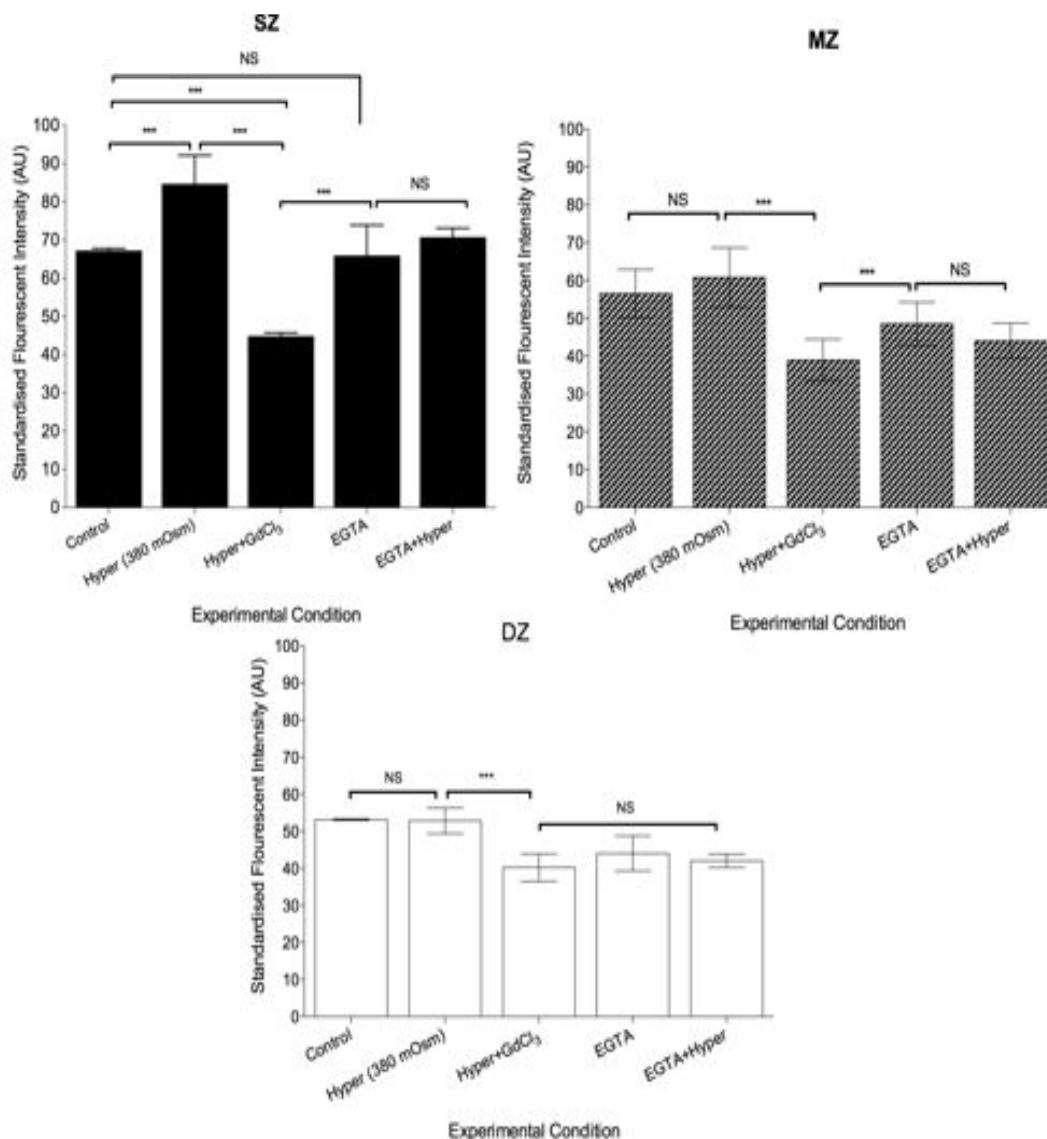
Figure 3.10 The role of the actin organization in chondrocyte protection.

*Cartilage explants were incubated in control and hypertonic DMEM (380 mOsm; 30 %) with and without the presence of 100 μM $GdCl_3$ or 2 mM EGTA. Explants were fixed with 4 % paraformaldehyde and the polymerised actin cytoskeleton labelled with Alexa 488-phalloidin. Linear profiling analysis was used to quantify F-actin. Data are mean +/- SEM of N=9 n=30, *p<0.05, **p<0.01, ***p<0.001 all vs. control.*

To further investigate the role of the actin cytoskeleton, in a separate series of experiments the above agents were used to observe the effect of changes on the F-actin cytoskeleton within freshly isolated bovine articular chondrocytes (Po). The results recorded resembled the behaviour of *in situ* chondrocytes within the SZ. With an increase of extracellular osmolarity by 30 % the polymerisation of the F-actin cytoskeleton significantly increased to 64.39 ± 9.39 AU ($p<0.001$) compared to control samples 51.68 ± 7.89 AU. Interestingly, increasing the extracellular osmolarity by 50 % and 90 % did not significantly increase the levels of F-actin compared to 30 % with values 62.80 ± 9.39 AU and 67.33 ± 9.80 AU respectively ($p>0.05$). These data therefore suggest that altering the extracellular osmolarity by 30 % is sufficient to re-model F-actin cytoskeleton in freshly isolated cells (Figure 3.11).

The addition of $GdCl_3$ and EGTA to freshly isolated cells (Figure 3.12) was recorded and in the presence of EGTA, 25 % of the cells exhibited increase in cortical actin ($p<0.001$) compared to control Po cells. The addition of increased extracellular osmolarity in EGTA treated cells did not change the results recorded and data recorded was not significantly different from EGTA only treated cells with value of 68.02 ± 9.51 AU. $GdCl_3$ treatment inhibited the cortical actin concentration with a 15 % ($p<0.001$) reduction in freshly isolated cells compared to *in situ* chondrocytes thus showing a similar behaviour to data accrued from cartilage explants (Figure 3.10). However, it should be noted that isolated cells behaved differently in presence of EGTA or when incubated in the presence of EGTA and hypertonic conditions ($p<0.05$) thus suggesting that the ECM may be slowing down the effects of EGTA for *in situ* explants (Figure 3.12).
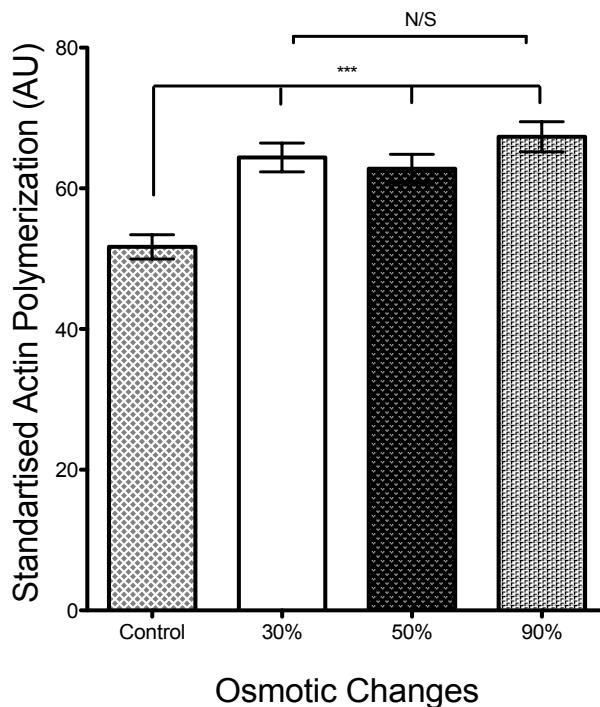
Figure 3.11 F-Actin organisation and the effect of Hyperosmotic pre-treatment on freshly isolated chondrocytes (Po).

*Freshly isolated cells (Po) were pre-treated for 1 h with control isotonic DMEM of 380 mOsm, or hypertonic DMEM of 500, 570 and 720 mOsm that represented 30 %, 50 % and 90 % increase of extracellular osmolarity respectively. Cells were fixed with 4 % paraformaldehyde and the polymerised actin cytoskeleton labelled with Alexa 488-phalloidin. Linear profiling analysis was used to quantify F-actin. Data are mean +/- SEM of n=30, *p<0.05, **p<0.01, ***p<0.001 all vs. control from three distinct experiments.*
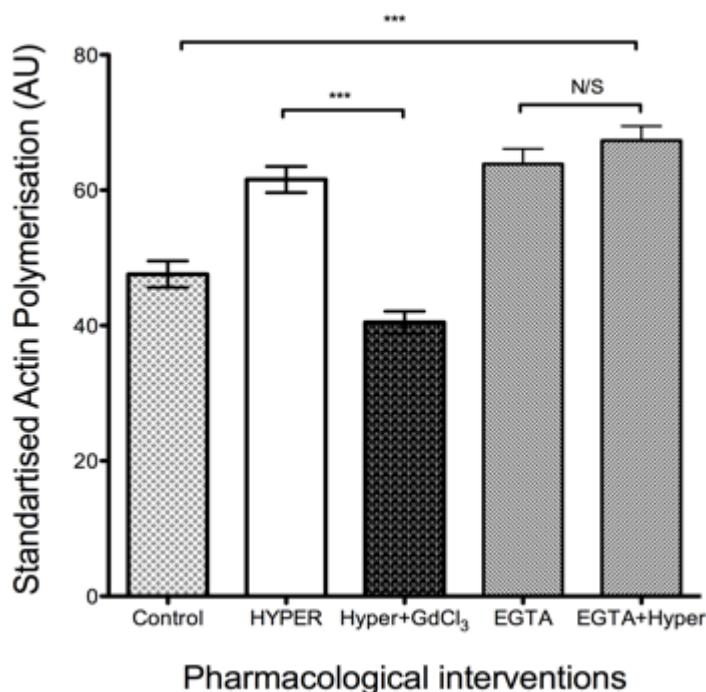
Figure 3.12 The role of actin organisation and the effect of cell shrinkage, gadolinium chloride and EGTA on freshly isolated chondrocytes (Po).

Freshly isolated cells (Po) were pre-treated for 1 h in control and hypertonic DMEM (380 mOsm; 30 %) with and without the presence of 100 μM GdCl$_3$ or 2 mM EGTA, fixed with 4 % paraformaldehyde. The polymerised actin cytoskeleton labelled with Alexa 488-phalloidin. Linear profiling analysis was used to quantify F-actin and following a hypertonic challenge. Data are mean +/- SEM of n=30, *p<0.05, **p<0.01, ***p<0.001 all vs. control from three distinct experiments.

3.3 Summary

Mechanical loading following a single impact caused a rapid initial cell death followed by a slower subsequent death within a short period of time (30 min). Single mechanical load on some occasions caused fissures within the cartilage explants thus compromising the integrity of the tissue. Formations of tissue lesions or fissures that compromise the integrity of tissue have been reported to increase the risk of development of secondary OA (Bader *et al.,* 2011, Goldring, 2012). Cell shrinkage following a hypertonic challenge of the medium reduced the rates of cell death in chondrocytes, post-mechanical impact and offered chondro-protective effect. Furthermore, hypertonic treatment resulted in an increase in F-actin polymerisation prior to impact and protected against cell death.

Interestingly, removal of extracellular calcium with EGTA was not shown to be chondro-protective (Amin *et al.,* 2009) within the first 30 min post impact, however *in situ* chondrocytes showed characteristics related with chondro-protection such as reduction of cell volume thus indicating that calcium influx might have a time-delayed effect. Treatment with $GdCl_3$ which blocks $[Ca^{2+}]_i$ (Erickson *et al.,* 2001, Lewis *et al.,* 2011) led to an exacerbation in cell death, even when the chondro-protective challenge was employed, thus indicating that the stretch-sensitive calcium channels were involved in affording chondro-protection. Potential explanations for this effect could be due to Young's Modules or perhaps channel activation.

Treatment of cells with Jasplakinolide (the F-actin stabilising agent) resulted in chondro-protection, indicating a role for F-actin, whilst Latrunculin B (the actin depolymerising agent) increased cell death, again drawing attention to the cell cytoskeleton and its role in chondro-protection.

Finally, the organisation of the actin cytoskeleton was evaluated and was observed that *in situ* and isolated cells respond in similar way to environmental changes. Additionally, it was observed that cells within characteristically different cartilage zones responded differently to

environmental changes. The increase in extracellular osmolarity, and thus chondro-protection following impact load, was the result of a polymerisation of the actin cytoskeleton that was dominant in the SZ zone alone and was inhibited by a pre-incubation with the stretch-sensitive calcium channel inhibitor $GdCl_3$.

CHAPTER 4

4. EXPERIMENTAL CHAPTER: REV5901 and isomers; and investigation of its Chondro-protective Actions.

4.1 Introduction

Whilst mechanical loading is recognised to be important and essential for maintaining healthy cartilage and a viable ECM (Wilkins, 2000, Kruz *et al.,* 2005), repetitive and excessive mechanical loading can cause hydrostatic stress, tensile strain and abnormal fluid shear stress leading to irreversible cartilage erosion and acute traumatic joint injury, recapitulating the hallmarks of OA (Felson *et al.,* 2000, Buckwalter *et al.,* 2006). As shown in Chapter 3, a blunt mechanical trauma led to a rapid cell death and a decrease in cortical actin. Previous studies have reported that injured cartilage has a limited capacity for repair and several studies have shown that excessive mechanical loading can trigger apoptosis and necrosis in chondrocytes as well as causing structural damage to cartilage (Borelli *et al.,* 1997, Jeffrey *et al.,* 1997, Chen *et al.,* 2001, D'Lima *et al.,* 2001a, Kurz *et al.,* 2001, Amin *et al.,* 2008).

Osteoarthritis (OA) is a degenerative disease marked by destruction of cartilage with multifactorial causes, but is generally strongly associated with age and mechanical aspects (Felson *et al.,* 2000, Buckwalter and Martin, 2004). Different studies have examined both the biochemical and morphological nature of the disease and shown a possible 'pre-osteoarthritic state' in relation to the mechanism of joint damage with overuse (Vasan, 1983, Brown *et al.,* 1984, Lequesne *et al.,* 1997, Buckwalter and Martin 2004, Felson and Kim, 2007). Mechanical impact or joint overuse has been reported to be responsible for cartilage fissures and lesions (Donohue *et al.,* 1983) and evidence exists that high impact sports e.g. football, show an increased risk of OA in knee and hip joints (Lindberg *et al.,* 1993, Roos *et al.,* 1994, Urquhart *et al.,* 2007, Dvorak, 2011). It is therefore important to investigate the protective mechanisms against mechanical loading and supplements that may afford chondro-protection as current treatments mainly target symptoms of the pathology e.g. pain. Recent studies have reported increased evidence in the role of the subchondral bone in OA, thus both articular cartilage and the subchondral bone are considered to be involved in

the initiation and progression of this pathology (Spector *et al.,* 2005, Iwamoto *et al.,* 2010). This has resulted in the interest of drugs that influence bone metabolism such as the promising chondro-protective agent pyridinyl bisphosphonate risedronate approved for clinical use since 2000 in the USA, for the treatment of osteoporosis (Van Offel *et al.,* 2005, Mauck and Clarke, 2006), and the tetracycline antibiotic doxycycline which has been shown to inhibit cartilage MMPs (Smith *et al.,* 1996, Shilopov *et al.,* 1999). However, neither has been associated with definitive results and effectiveness has been questioned due to large heterogeneity between studies (Nüesch *et al.,* 2009, Da Costa *et al.,* 2012). In addition to this, some safety concerns have been raised with the long-term use of doxycycline (Dieppe, 2005, Da Costa *et al.,* 2012). Therefore, the need to identify novel chondro-protective agents is paramount and in this study, the RVD inhibitors, REV 5901 and L-665, 238 (REV 5901 para-isomer) have been investigated for their potential role to uncover a chondro-protective pathway.

4.1.1 Chondro-protective agents

REV 5901 (a-pentyl-3-(2-quinolinylmethoxy)-benzene-methanol is a 5-lipoxygenase inhibitor (McMillan and Walker, 1992, Bush and Hall, 2001), an antagonist of a family of G-protein coupled receptors (the cystenyl-leukotriene receptors; CYS-LT) in porcine epithelial cells (Van Inwegen *et al.,* 1987) and an inhibitor of RVD (Hall *et al.,* 1998, Qusous *et al.,* 2012). Since targeting volume changes that have been reported to be important for chondro-protection (Clark *et al.,* 2010), the effect of REV 5901 was investigated as a potential novel therapeutic chondro-protective agent.

The para-isomer of REV5901 (L-655,238) was also investigated and is a potent and selective inhibitor of 5-lipoxygenase activating protein (FLAP; Evans *et al.,* 1991). However, despite the similarity with REV 5901, L-655,238 has reported differing results regarding its efficacy, with studies highlighting that although its a potent and selective inhibitor of FLAP (Ford-Hutchinson, 1991), it does not exhibit the same range of properties as REV 5901 and thus its effects were investigated here.

4.1.2 Chapter aims

This study aims to investigate the chondro-protective properties of a panel of pharmacological compounds determining their effects on:

1. Short term post- mechanical impact on chondrocyte viability and volume;
2. Short term post-mechanical trauma;
3. Investigation of the involvement of a known pathway in the potential mechanism of chondro-protection;

4.2 Results

4.2.1 REV 5901: the protective effect on bovine articular cartilage following mechanical impact.

After confirming the initial osmolarity of DMEM using a Vapro^{TM} pressure osmometer to be 280.71 ± 0.70 mOsm, the osmolarity of DMEM with the addition of REV 5901 and DMSO (vehicle for REV 5901) was determined with an osmolarity of 281.00 ± 0.70 mOsm and 281.14 ± 1.76 mOsm respectively observed, non-significant compared to DMEM osmolarity alone (Figure 4.1).

Following identification that medium osmolarity was not altered by addition of DMSO (drug vehicle), the effects on cell volume and viability were investigated, using Imaris 7.1 'Iso surface' or 'Spots' as previously described (Bush *et al.,* 2005, Kerrigan *et al.,* 2005, Nedelcheva *et al*., 2008). Following addition of REV 5901, cell volume changed significantly compared to control isotonic conditions (280 mOsm) decreasing from 716.14 ± 36.68 µm^3 to 552.68 ± 24.38 µm^3 (p<0.001) respectively, with a decrease of 23 %. These data show similar trends to volume changes recorded for *in situ* chondrocytes incubated in hypertonic conditions (380 mOsm) observed in chapter 3, 578.46 ± 26.85 µm^3 (p>0.05; Figure 4.2).

Following identification of the effects on cell volume, the effects of REV 5901 on cell viability were subsequently investigated. REV 5901 treatment displayed no detrimental effect on chondrocytes or cartilage. Explants pre-treated for 1 h with REV 5901 (50 μM) have experienced a reduction in PDC by 51 % to 5.44 ± 0.59 % (p<0.001) compared to the control sample where impact led to an 11.23 ± 1.24 % cell death 30 min post impact. When cell death was examined in samples treated only with vehicle (DMSO, 0.1 %) loss of cell viability 30 min post impact was recorded as 9.20 ± 0.65 % (p>0.05), compared to control samples (Figure 4.3) thus indicating that DMSO cannot be responsible for the increased cell viability of cells treated with REV 5901.
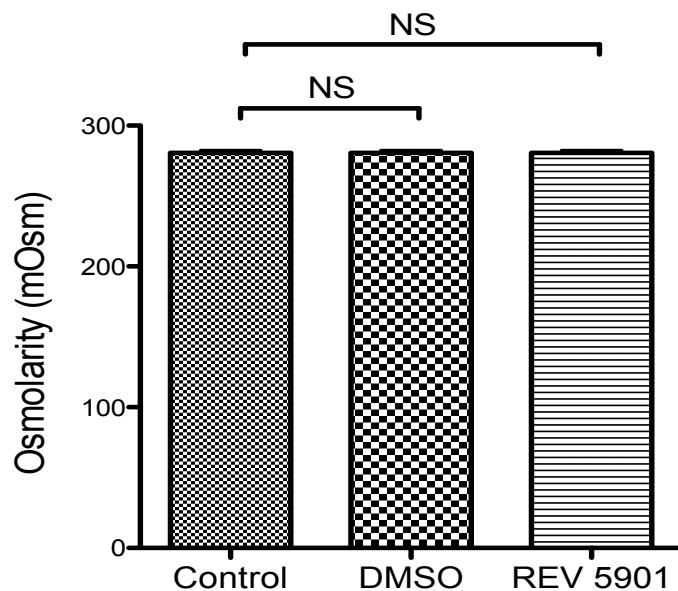
Figure 4.1 REV 5901 has no effect on medium osmolarity.

Using Vapro^TM vapour pressure osmometer samples of control isotonic DMEM, DMSO (0.1 %) and REV 5901 (50 μM) supplemented DMEM were measured for osmolarity. The addition of nether DMSO or REV 5901 was observed to significantly alter medium osmolarity. N=20

(A)

| CONTROL | DMSO | HYPER | REV 5901 |

(B)
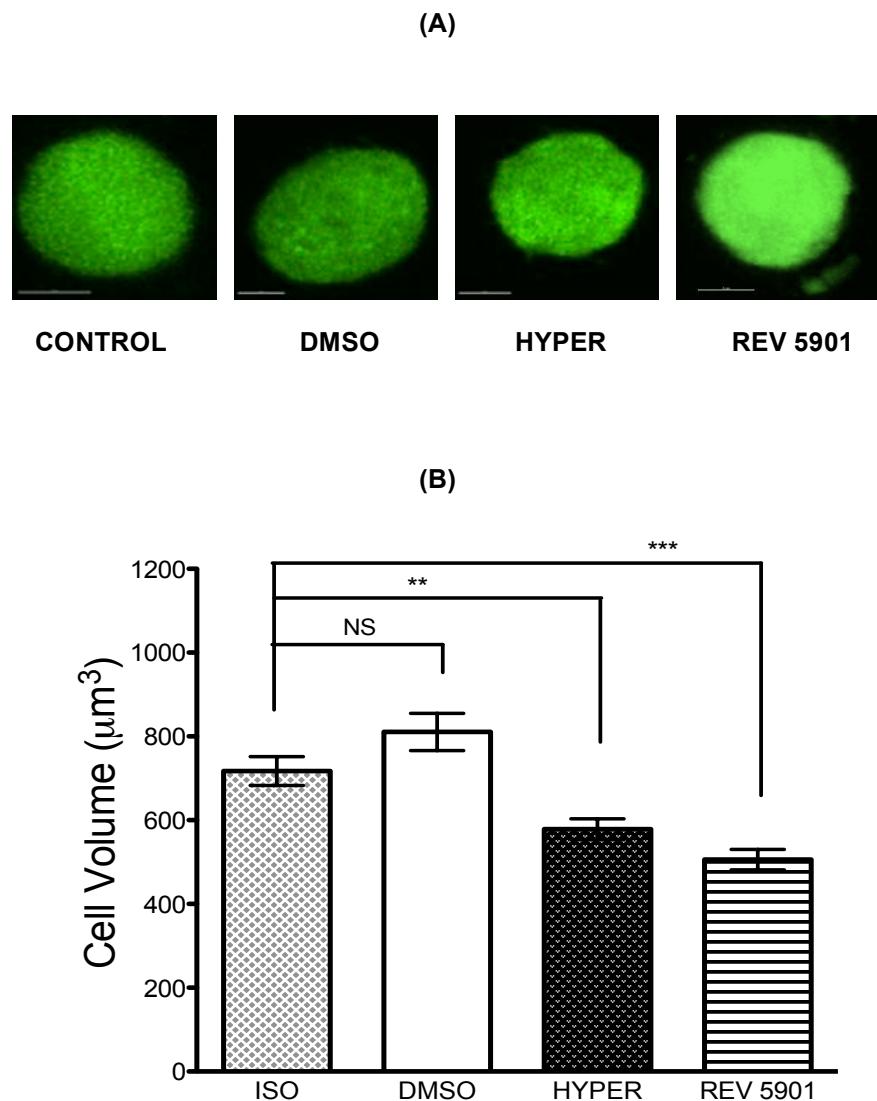
Figure 4.2 REV 5901 decreases cell volume.

Bovine articular cartilage explants were pre-treated for 1 h with control isotonic, hypertonic (380 mOsm) DMEM or DMSO (0.1 %) or REV 5901 (50 μM) supplemented DMEM. Samples were loaded with Calcein AM (5 μM) and images were acquired with CLSM post treatment (A) using a x63 dipping objective imaging 'top-down' from the articular surface. To quantify cell volume 'Iso surface' volume analyses were used and REV 5901 was observed to significantly decrease cell volume (B). N=9, n=40 cells, *p<0.05, **p<0.01, ***p<0.001 all vs. control from three distinct experiments. Bar in (A) = 5 μm
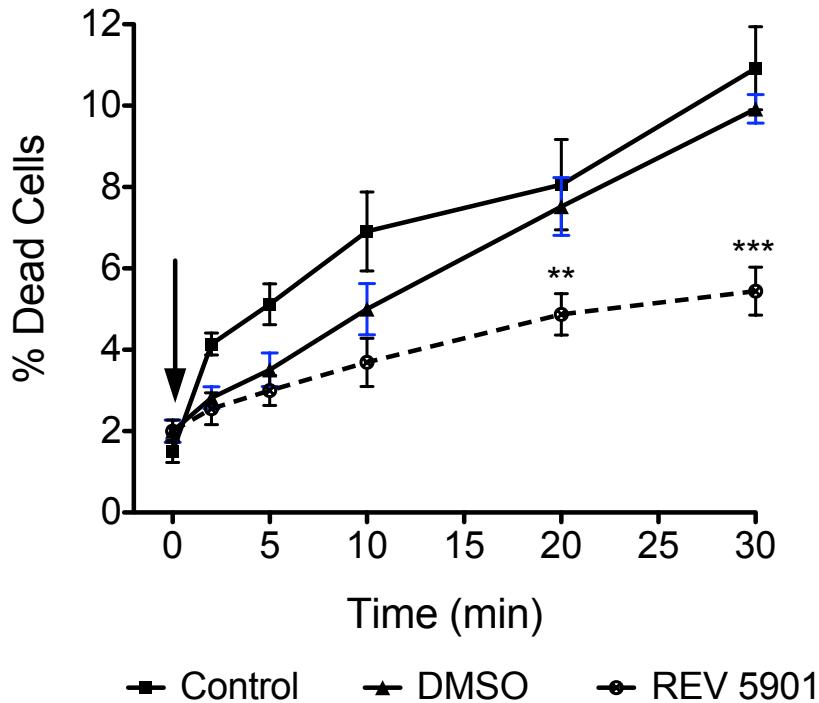
Condition	Viability at 2 min (%)	Viability at 30min (%)
Control (280)	95.86 ± 0.27	89.08 ± 1.02
DMSO	97.18 ± 0.27	90.08 ± 0.35
REV 5901	97.45 ± 0.39	94.56 ± 0.59 ***

Figure 4.3 Pre-treatment with REV 5901 protects against cell death post impact.

Bovine articular cartilage explants were pre-treated for 1 h with control isotonic, hypertonic (380 mOsm) DMEM, DMSO (0.1 %) or REV 5901 (50 μM) supplemented DMEM. Samples were loaded with Calcein AM (5 μM) and PI (2 μM) subject to single impact (10 cm) and images were acquired with CLSM prior and post impact at 2, 5, 10, 20 and 30 min. To quantify cell death Imaris spot analysis were used. REV 5901 was observed to protect against cell death. N=9, n=27 explants, *p<0.05, **p<0.01, ***p<0.001 all vs. control from three distinct experiments. Arrow denotes impact point.

4.2.2 The effect of the L-655,238 (REV 5901 para-isomer) on articular cartilage explant cells.

The potent and selective inhibitor of FLAP, L-655,238 (REV 5901, para-isomer) was used in order to help identify whether the isomer or the active form would be a more potent inhibitor of chondrocyte cell death. The osmolarity of L-665,238 supplemented DMEM was investigated to rule out the possibility that its effects were *via* osmotic mechanisms where DMSO was used as a vehicle. L-665,238 did not significantly alter the osmolarity medium with osmorarity of 280.86 ± 1.68 mOsm compared to control DMEM and DMSO supplemented medium, 280.71 ± 0.70 mOsm and 281.14 ± 1.76 mOsm respectively (Figure 4.4).

Cell shrinkage has previously been determined to be chondro-protective (Bush *et al.,* 2005, Lewis *et al.,* 2007) and treatment of cartilage explants with REV 5901 (50 µM) caused this (Figure 4.3), given this observation the effect of L-655,238 was determined. L-655,238 (50 µM) significantly decreased cell volume from 716.14 ± 36.68 μm^3 in control samples to 513.54 ± 23.15 μm^3 a reduction of 28% (p<0.001). When compared to the shrinkage caused by REV 5901 (552 ± 24.38 μm^3) it was found that this reduction of *in situ* chondrocyte cell volume was similar to that observed with REV 5901 treatment (p>0.05). DMSO supplemented DMEM used as a control caused an increase in cell volume and thus the effects of the drug were not due to the vehicle used hence, data was consistent with our previous findings (Figure 4.5).

Following a 10 cm impact delivered by a drop tower, chondrocyte death in explants incubated with L-655,238 supplemented DMEM conveyed an interesting pattern. Results recorded for the first 15 min were similar to DMSO and REV 5901 incubated samples with no significant differences in cell death for chondrocytes incubated in L-655,238 compared to control samples. Twenty min post impact data for L-655,238 incubated samples showed a similar response to REV 5901 incubated explants and were significantly different from control 4.95 ± 0.51 and 4.87 ± 0.53, p<0.01

respectively. Lastly 30 min post impact PDC was recorded to be 7.31 ± 0.59 % in L-655,238 treated cells compared to control samples where PDC was 10.92 ± 1.02 % thus showing a significant reduction (p<0.05). Interestingly, when comparing REV 5901 to L-655,238 it was found that REV 5901 demonstrated a greater chondro-protective effect. Significantly decreasing cell death by 51 % (p<0.001) at 30 min post impact compared to only 5 % (p<0.05) for L-655,238 at 30 min post impact with protective effect diminishing over time (Figure 4.6 A & B), thus suggesting that L-655,238 does not possess the chondro-protective potential seen by REV 5901.
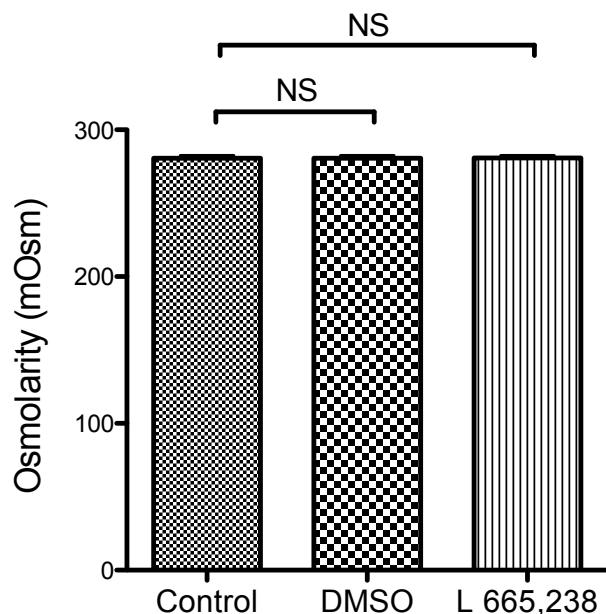
Figure 4.4 L-655,238 (REV 5901 para-isomer) has no effect on medium osmolarity.

Using Vapro^TM vapour pressure osmometer samples of control isotonic DMEM, DMSO (0.1 %) and L-665, 238 (50 μM) supplemented DMEM were measured for osmolarity. The addition of nether DMSO or L-665,238 were observed to significantly alter medium osmolarity. N=20

(A)

| CONTROL | DMSO | REV 5901 | L-665,238 |

(B)
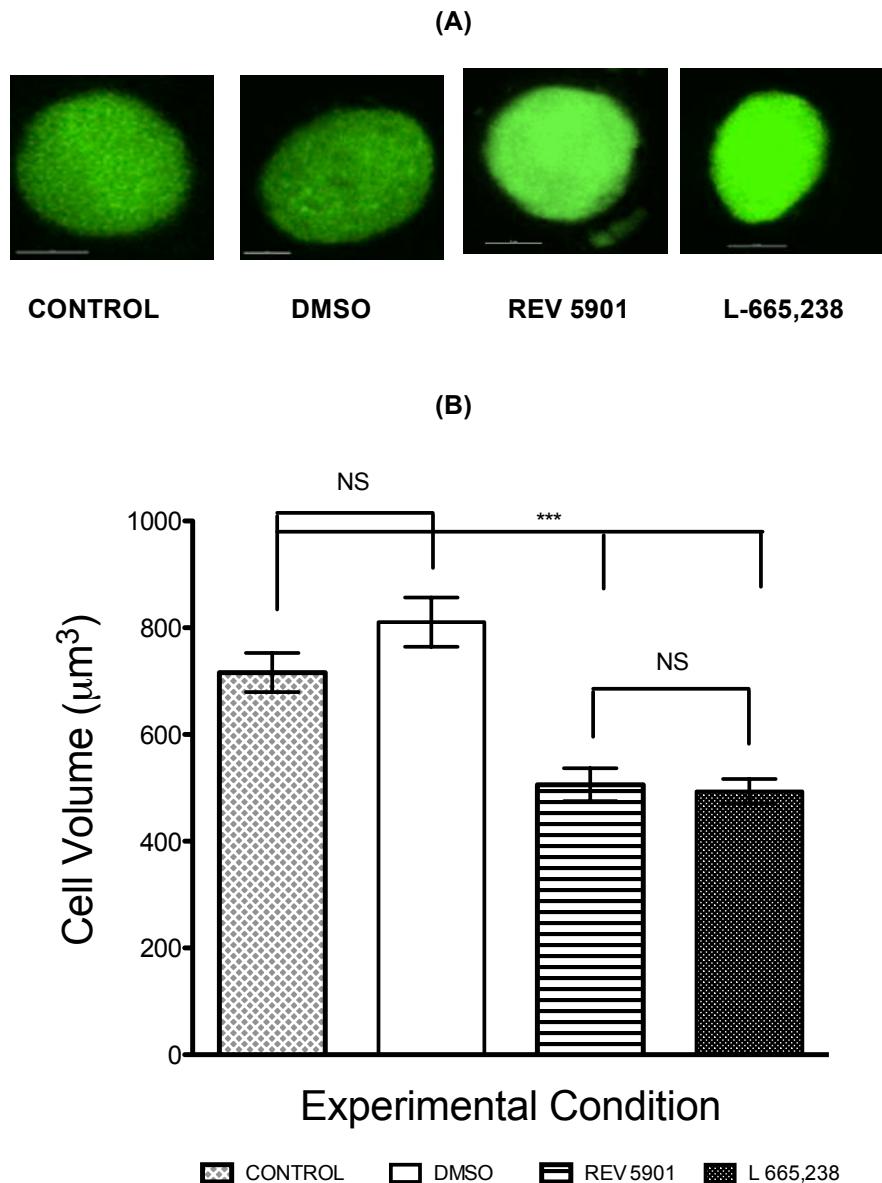
Figure 4.5 L-655,238 (REV 5901 para-isomer) and its effect on cell volume.

Bovine articular cartilage explants were pre-treated for 1 h with control isotonic DMEM, DMSO (0.1 %), REV 5901 (50 μM) or L-665,238 supplemented DMEM. Samples were loaded with Calcein AM (5 μM) and images were acquired with CLSM post treatment (A), using a x63 dipping objective imaging 'top-down' from the articular surface. To quantify cell volume 'Iso surface' volume analyses were used (B). Data are mean +/- SEM of N=9, n=40 cells, *p<0.05, **p<0.01, ***p<0.001 all vs. control from three distinct experiments. Bar = 5 μm

(A)

(B)
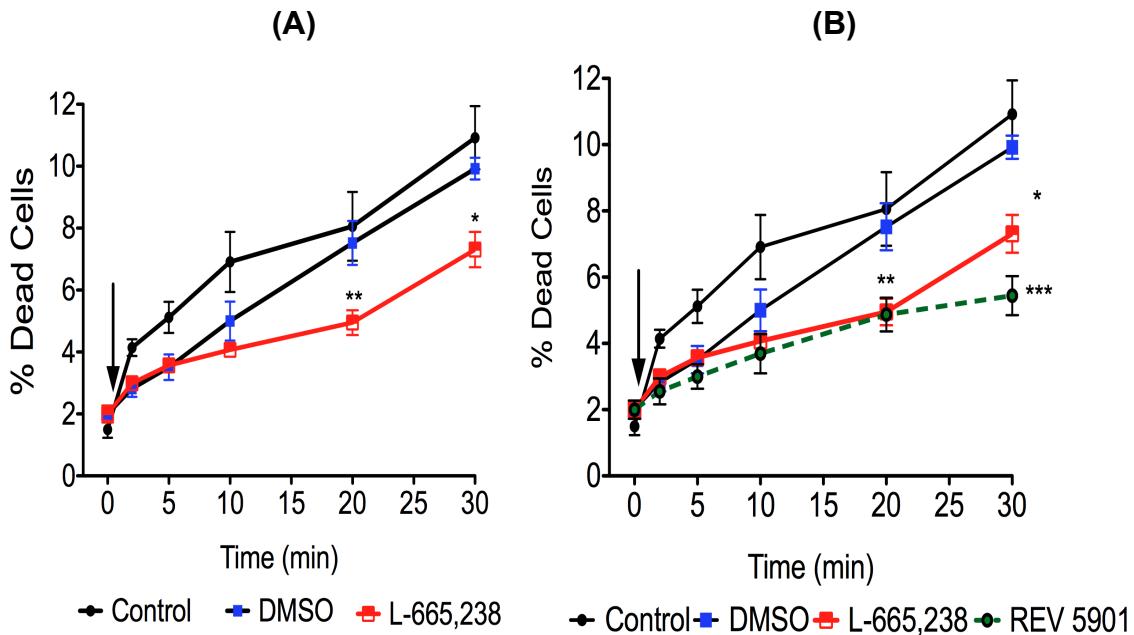
Condition	Viability at 2 min (%)	Viability at 30min (%)
Control (280)	95.86 ± 0.27	89.08 ± 1.02
DMSO	97.18 ± 0.27	90.08 ± 0.35
L-665,238	97.45 ± 0.07	91.69 ± 0.59 *
REV 5901	97.45 ± 0.39	94.56 ± 0.59 ***

Figure 4.6 L-655,238 (REV 5901 para-isomer) and its effect on *in situ* bovine articular chondrocytes viability.

Bovine articular cartilage explants were pre-treated for 1 h with control isotonic DMEM, DMSO (0.1 %), L-665,238 (A) (REV 5901-paraisomer; 50 μM) or REV 5901 (50 μM) (B) or supplemented DMEM. Samples were loaded with Calcein AM (5 μM) and PI (2 μM) subject to single impact (10 cm) and images were acquired with CLSM prior and post impact at 2, 5, 10, 20 and 30 min. To quantify cell death Imaris spot analysis were used. Arrow denotes impact point. Data are mean +/- SEM of N=9, n=27 explants, *p<0.05, **p<0.01, ***p<0.001 all vs. control from three distinct experiments.

4.2.3 Wortmannin and its effect on volume regulation.

To try and dissect the exact mechanism of action of REV 5901, a series of pharmacological inhibitors was used to investigate the effect of these compounds on different cell signaling cascades. Wortmannin a fungal metabolite and specific inhibitor of phosphatidylinositol 3-kinase (PI3K), mitogen-activated protein kinase (MAPK) and myosin light-chain kinase (MLCK) has been extensively used to demonstrate the role of the PI3K in diverse signal transduction processes (Stain and Waterfield, 2000). Wortmannin causes apoptotic cell death (Qureshi *et al.,* 2007) and inhibits cell volume recovery in different cells including PT3 (prostate cancer cell line), HTC (rat hepatoma cells) and U937 (human monocyte - macrophage cells) (Feranchak *et al.,* 1998). Since inhibition of PI3K delays cell volume recovery (Otero *et al.,* 2005, Lewis *et al.,* 2011), wortmannin's effects on REV 5901 inhibition of cell volume and protective effects on cell viability were determined.

The osmolarity of wortmannin supplemented DMEM was investigated to rule out the possibility of it acting *via* osmotic mechanisms and it was observed that wortmannin did not significantly adjust the osmolarity medium 280.86 ± 1.68 mOsm compared to control 280.71 ± 0.70 mOsm or the REV 5901 supplemented medium (Figure 4.7).

Cell shrinkage has previously been shown to be chondro-protective and therefore the effect of wortmannin was determined. In Chapter 3, cell shrinkage displayed a chondro-protective effect but when the cells were shrunk below 440.13 ± 16.42 μm^3 cell viability post impact did not show significant changes compared to control samples (Figure 3.3). Cells incubated within wortmannin (10 nM) supplemented DMEM exhibited a significant decrease in cell volume from 716.14 ± 36.68 μm^3 in control samples to 359.90 ± 12.23 μm^3 (p<0.001); a decrease of 50 %. Cells pre-treated with REV 5901 (50 µM) displayed a cell volume of 552.68 ± 24.38 μm^3 a reduction of 30 % compared to controls (p<0.01). However, when cells were treated with a combination of REV 5901 (50 µM) and wortmannin, there

was no synergistic effect observed compared to treatment of cell alone with each agent. In fact the combination treatment led to chondrocytes exhibiting the same volume as REV 5901 alone (Figure 4.8).

Cell death was significantly increased following a single impact. Explants treated with wortmannin (10 nM) led to an increase in cell death as observed at 2 min post impact, where cell viability was reduced to 91.98 ± 0.41 % compared to 95.86 ± 0.27 % in control samples (p<0.001) The significant increase in cell death continued over the time-period such that at 30 min post impact, cell viability was reduced by an additional 8 % compared to control samples (p<0.001) and by 10 % compared to REV 5901 incubated samples (p<0.001). Interestingly, when a combination of REV 5901 (50 µM) and wortmannin was investigated, cell viability was restored to levels seen in control sample thus showing that protective effect of REV 5901 was inhibited by wortmannin. No significant difference in cell death measurements were recorded prior to impact trauma for explants incubated in wortmannin compared to control samples (Figure 4.9 A & B).
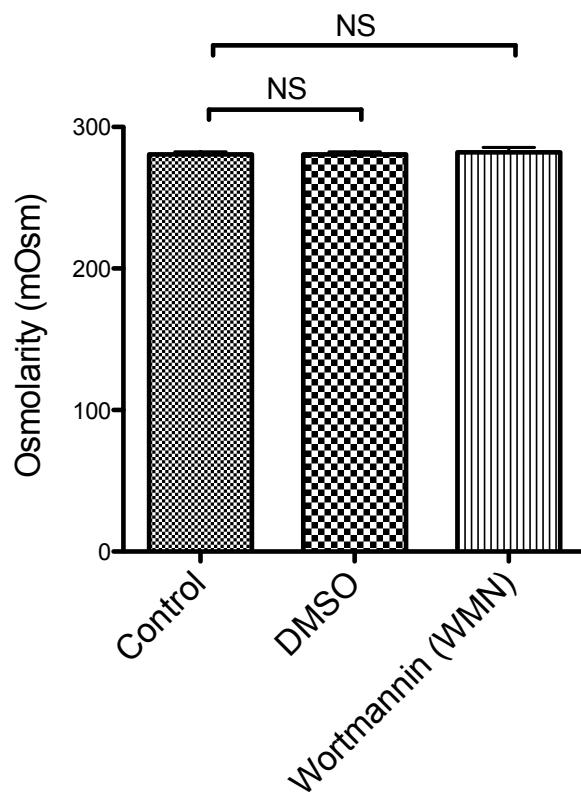
Figure 4.7 Wortmannin and its effect on medium osmolarity.

Using Vapro^{TM} vapour pressure osmometer samples of control isotonic DMEM, DMSO (0.1 %) and Wortmannin (10 nM) supplemented DMEM were measured for osmolarity. The addition of neither DMSO or wortmannin were observed to significantly alter medium osmolarity. N=20

(A)

| CONTROL | REV 5901 | Wortmannin | WMN + REV 5901 |

(B)
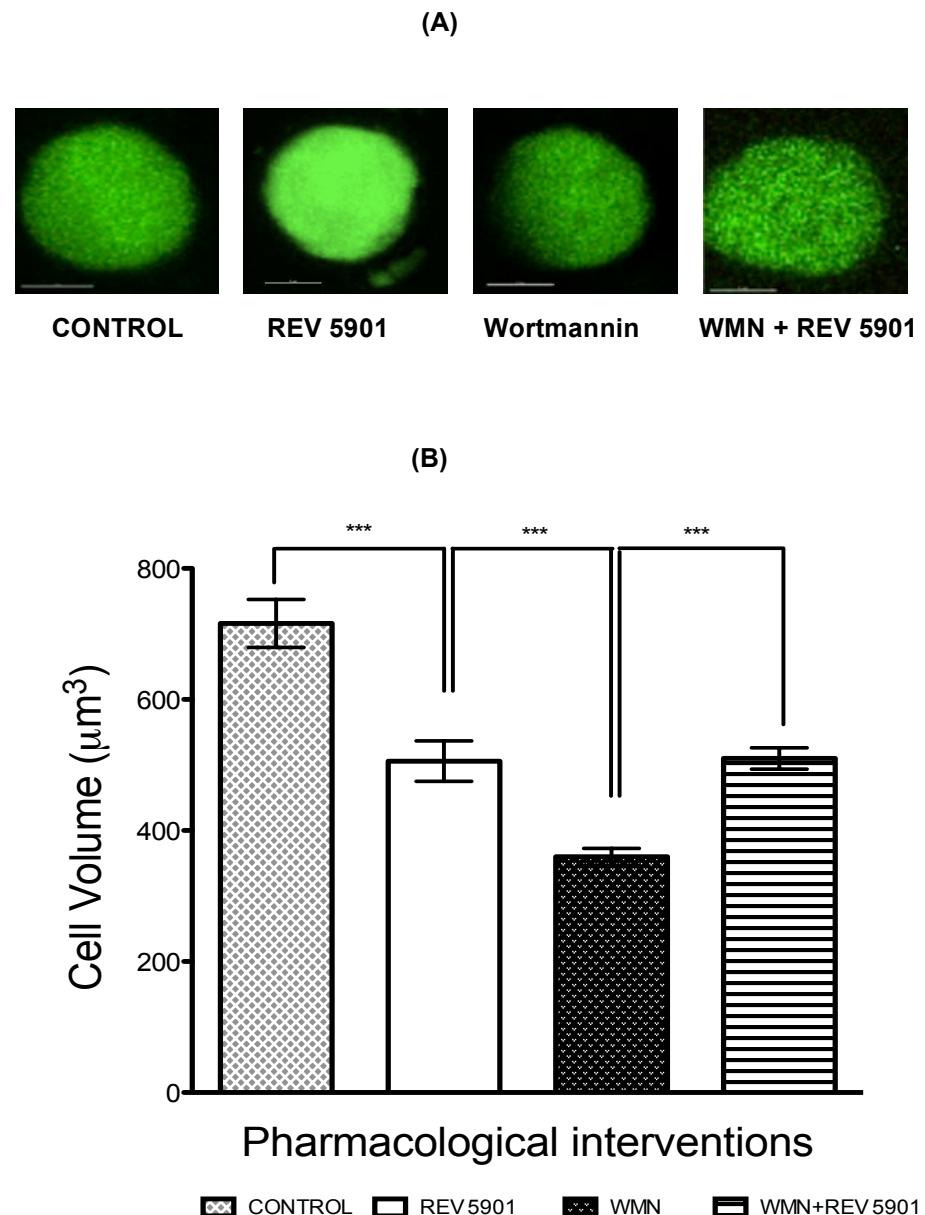
Figure 4.8 The effect of Wortmannin and REV 5901 on *in situ* bovine cell volume.

Bovine articular cartilage explants were pre-treated for 1 h with control isotonic DMEM, REV 5901 (50 μM), wortmannin (WMN) (10 nM) or wortmannin (WMN) and REV 5901 supplemented DMEM. Samples were loaded with Calcein AM (5μM) and images were acquired with CLSM post treatment (A) with a x63 dipping objective imaging the zone of cartilage 'top-down' from the articular surface. To quantify cell volume 'Iso surface' volume analyses were used (B). Data are mean +/- SEM of N=9, n=40 cells, *p<0.05, **p<0.01, ***p<0.001 from three distinct experiments. Bar =5 μm

(A)

(B)
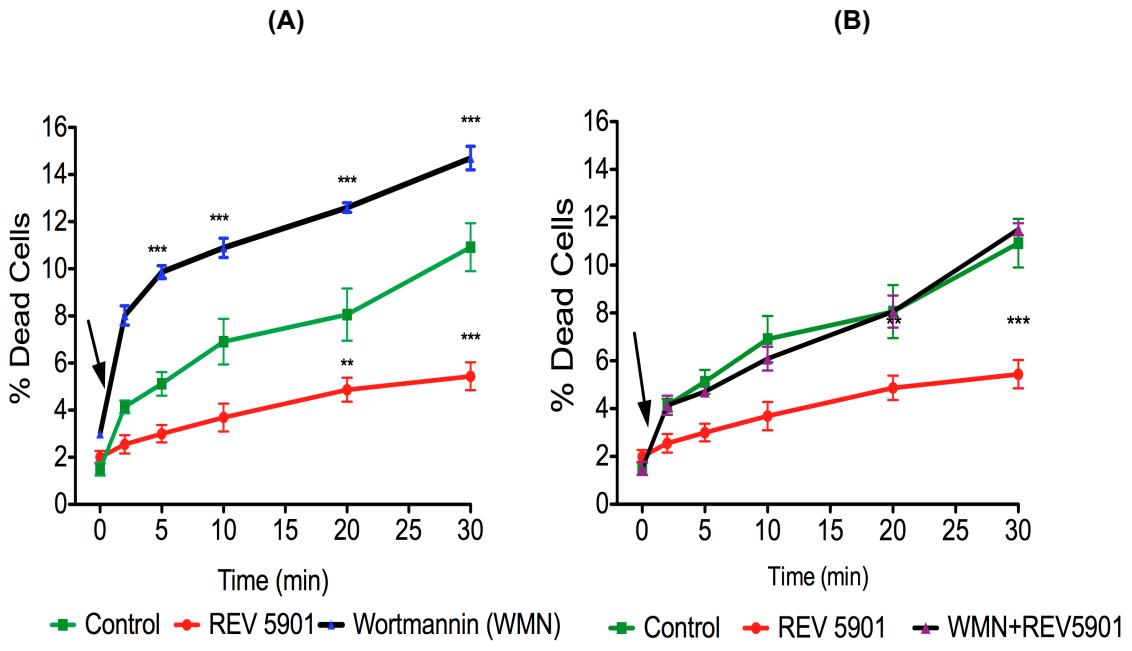
Condition	Viability at 2 min (%)	Viability at 30min (%)
Control (280 mOsm)	95.86 ± 0.27	89.08 ± 1.02
REV 5901	97.45 ± 0.39	94.56 ± 0.59 ***
Wortmannin (WMN)	91.98 ± 0.41***	85.3 ± 0.50 ***
WMN + REV 5901	95.85 ± 0.40	88.50 ± 0.25

Figure 4.9 Pre-treatment with Wortmannin and REV 5901 on cell viability.

*Bovine articular cartilage explants were pre-treated for 1 h with control isotonic DMEM, REV 5901 (50 μM), wortmannin (10 nM) or wortmannin (WMN) and REV 5901 supplemented DMEM. Samples were loaded with Calcein AM (5 μM) and PI (2 μM) and subject to single impact (10 cm) and images were acquired with CLSM prior and post impact at 2, 5, 10, 20 and 30 min. To quantify cell death Imaris spot analysis were used. Data are mean +/- SEM of N=9, n=27 explants, *p<0.05, **p<0.01, ***p<0.001 all vs. control from three distinct experiments. Arrow denotes impact point.*

4.2.4 Determining whether Calcium Signalling plays a role in the mechanism of action of REV 5901 in short term mechanical trauma.

Calcium signaling forms part of a possible mechanotransduction pathway by which chondrocytes may alter their metabolism in response to mechanical loading (Lewis *et al.,* 2007). The effect on cell shrinkage on chondro-protection and the role of stretch activated calcium channel (SACC) was determined in Chapter 3. Treatment with $GdCl_3$, an inhibitor of SACC, exacerbated the effect of cell death even when the chondro-protective osmotic challenge was employed thus indicating that the SACC were involved in affording chondro-protection. Therefore, the chondroprotection offered by REV 5901 was tested in the presence of $GdCl_3$ to determine whether its effects were via SACC.

Bovine articular cartilage explants were pre-treated for 1 h with control isotonic, hypertonic (380 mOsm) DMEM or $GdCl_3$ (100 μM) and hypertonic (380 mOsm) DMEM, or REV 5901 (50 μM), or REV 5901 (50 μM) with $GdCl_3$ (100 μM) supplemented DMEM and cell viability determined post impact at 2, 5, 10, 20 and 30 min. Within the first two minutes post impact samples incubated with $GdCl_3$ and stimulated with REV 5901 were observed to offer an enhanced protection against cell death that was significantly greater (95.36 $\pm$ 0.26 %) compared to samples with $GdCl_3$ (89.33 $\pm$ 1.13 %, $p<0.05$) + hyperosmotic DMEM (380 mOsm), data recorded for REV 5901 treated samples was 97.45 $\pm$ 0.39 %. However, with time progression post impact, at 30 min cell death levels in samples pre-incubated with $GdCl_3$ and stimulated with REV 5901 increased and shown to be significantly higher than control samples ($p<0.01$) and samples incubated with REV 5901 alone ($p<0.001$; Figure 4.10). These data suggests that the chondro-protective effect of REV5901 was abolished by $GdCl_3$ thus pointing out the SACC as possible involvement.
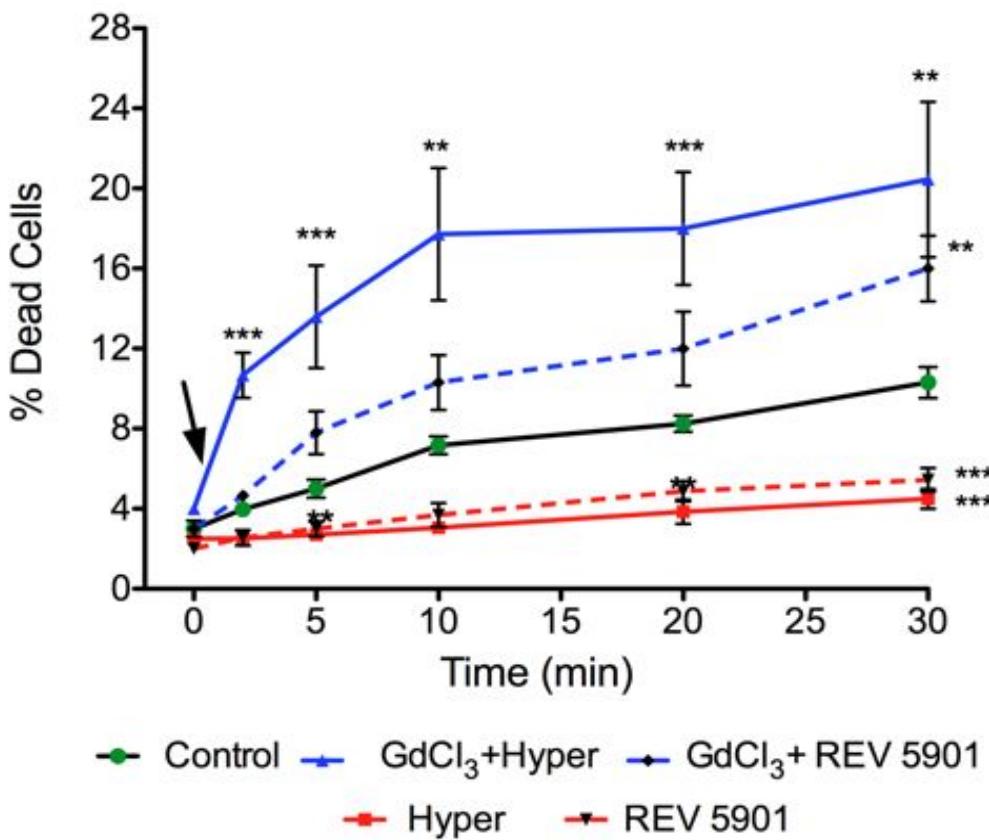
Experimental Condition	Viability at 2 min (%)	Viability at 30min (%)
Control (280 mOsm)	95.86 ± 0.27	89.08 ± 1.02
GdCl$_3$ + Hyper (380mOsm)	89.33 ± 1.13 ***	79.55 ± 3.87 ***
GdCl$_3$+ REV 5901	95.36 ± 0.26	85.17 ± 1.64 **
Hyper (380 mOSm)	97.48 ± 0.30	95.50 ± 0.49 ***
REV 5901	97.45 ± 0.39	94.56 ± 0.59 ***

Figure 4.10 REV 5901 and GdCl$_3$ the effect on cell viability within cartilage explants.

Bovine articular cartilage explants were pre-treated for 1 h with control isotonic, hypertonic (380 mOsm) DMEM or GdCl$_3$ (100 μM) and hypertonic (380 mOsm) DMEM, or REV 5901 (50 μM), or REV 590 (50 μM) with GdCl$_3$ (100 μM) supplemented DMEM. Samples were loaded with Calcein AM (5 μM) and PI (2 μM) and subject to single impact (10 cm) and images were acquired with CLSM prior and post impact at 2, 5, 10, 20 and 30 min. To quantify cell death Imaris spot analysis were used. Arrow denotes impact point. Data are mean +/- SEM of N=9, *p<0.05, **p<0.01, ***p<0.001 all vs. control from three distinct experiments.

Calcium mobilising mediators such as uridine 5'-triphosphate have been reported to cause a rise in intracellular calcium concentration ($[Ca^{2+}]_i$) in human articular chondrocytes (Koolpe *et al.,* 2005). Therefore, it was investigated if uridine 5'-triphosphate would have a positive effect on cell viability and act in a similar manner to REV 5901 or exhibits no effect on cell mechanotransduction and viability, thus providing further evidence of the pathway involved.

A significant decrease in the volume of *in situ* chondrocytes treated with uridine 5-'triphosphate (100 μM) was observed compared to control chondrocytes with a volume of 604.08 ± 24.33 μm^3 compared to 716.14 ± 36.68 μm^3 ($p < 0.05$) respectively, a 15 % decrease. However, chondrocyte cell volume following treatment with REV 5901 (50 μM) was still significantly lower than that observed with uridine 5'-triphosphate (100 μM; $p < 0.05$).

When analysing cell viability following incubation with uridine 5'-triphosphate (100 μM) it was observed that there were no significant changes in cell death levels compared to control within the first 20 min post impact ($p > 0.05$), however at later time-points a decrease in PDC was observed for uridine treated samples with 91.78 ± 0.57 % compared to 89.08 ± 1.02 % ($p > 0.05$) in control explants. This data shows that Ca^{2+} has once again been implicated to have an effect on chondrocyte cell volume (section 3.2.4 EGTA treated samples) but no effect over chondrocyte viability within 30 min post impact.

(A)   (B)
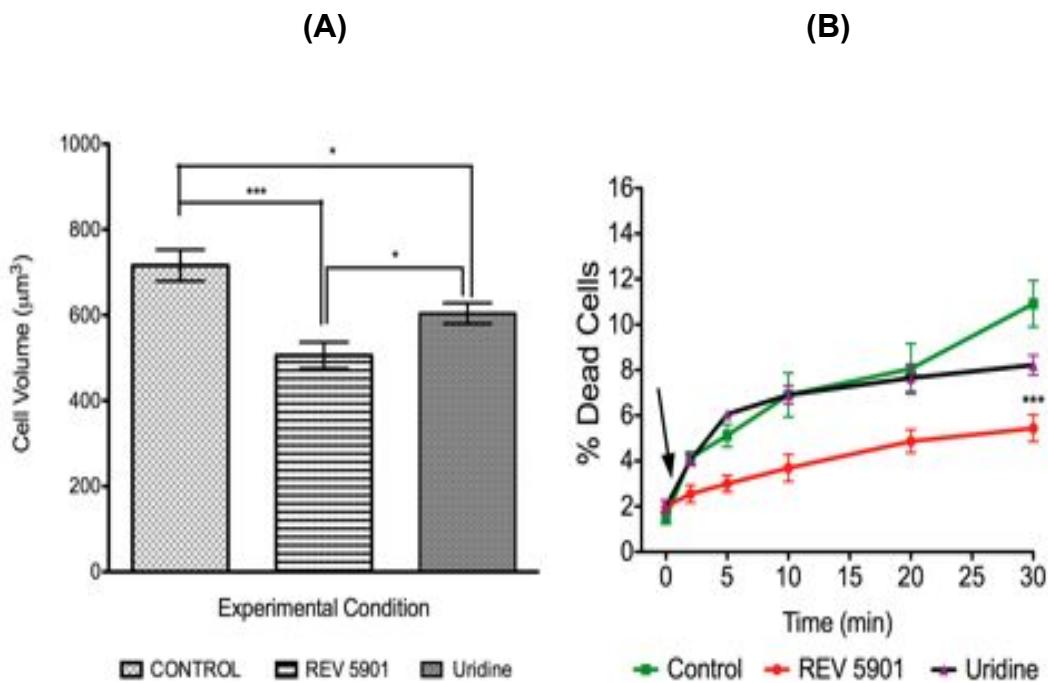
Experimental Condition	Chondrocyte Volume Changes (μm^3)	Viability at 2 min (%)	Viability at 30min (%)
Control (280mOsm)	716.14 ± 36.68	95.86 ± 0.27	89.08 ± 1.02
Uridine	578.46 ± 26.85 *	95.65 ± 0.37	91.78 ± 0.57
REV 5901	552.68 ± 24.38 **	97.45 ± 0.39	94.56 ± 0.59 ***

Figure 4.11 REV 5901 and Uridine: the effect on cell volume and viability within *in situ* articular chondrocytes.

Bovine articular cartilage explants were pre-treated for 1 h with control isotonic DMEM, REV 5901(50 μM), and Uridine (100 μM) supplemented DMEM. Panel A: Samples were loaded with Calcein AM (5 μM) and images were acquired with CLSM post treatment. To quantify cell volume 'Iso surface' volume analyses were used all pharmacological agents. Panel B: Samples were loaded with Calcein AM (5 μM) and PI (2 μM) and subject to single impact (10 cm) and images were acquired with CLSM prior and post impact at 2, 5, 10, 20 and 30 min. To quantify cell death 'Imaris spot analysis' were used. Arrow denotes impact point. Data are mean +/- SEM of N=9, n=40 cells, n=27 explants, *p<0.05, **p<0.01, ***p<0.001 all vs. control from three distinct experiments.

4.2.5 F-actin changes due to pharmacological interventions

Previous studies have shown that the actin cytoskeleton plays a vital role in maintaining cell structure and the integrity of bovine articular chondrocytes (Gulilak *et al.,* 1995, Guilak, 1999, Blain, 2009). Disruption of F-actin cytoskeleton with pharmacological agents have been reported to have negative effect on the relationship between matrix deformation and alterations in chondrocyte nucleus shape as well as a decrease chondrocyte stiffness (Gulilak *et al.,* 1995, Tickery *et al.,* 2004). Nevertheless, filamentous actin organisation has been noted to be less defined in OA suggesting a defective function of F-actin in OA (Blain, 2009). Therefore, it was important to investigate the effect of REV 5901 upon F-actin cytoskeleton.

REV 5901 induced increase in the actin cytoskeleton within the SZ with 78.72 ± 3.99 AU (p<0.01) compared to SZ cells from control samples (65.43 ± 3.99 AU). No significant differences were observed in the MZ, 61.17 ± 4.25 AU for REV 5091 samples and 56.49 ± 8.98 AU (p>0.05) in control samples or in the DZ, 47.50 ± 2.12 AU and 53.13 ± 1.00 AU respectively (p>0.05). DMSO was noted not to alter F-actin organisation thus confirming that the chondro-protective effects of REV 5901 are independent from the vehicle used (Figure 4.12 B).

F-actin organisation of *in situ* chondrocytes showed no significant changes in response to L-655,238 (REV 5901 para-isomer), regardless of cell zone location when compared to control samples (p>0.05; Figure 4.13). Data for SZ cells incubated in L-655,238 (50 µM) supplemented DMEM was 70.50 ± 10.83 AU compared to 65.43 ± 3.99 AU in control samples. Similar trends were observed for the MZ cells (52.88 ± 7.63 AU vs 56.49 ± 8.98 AU control) or DZ cells (51.63 ± 36.39 AU vs 53.12 ± 1.00 AU control) thus indicating that L-655,238 (REV 5901 para-isomer) has no effect on cell cytoskeleton and perhaps explaining why it was less effective at preventing cell death over the time course investigated observed in section 4.2.1 (Figure 4.6).

(A)

(B)
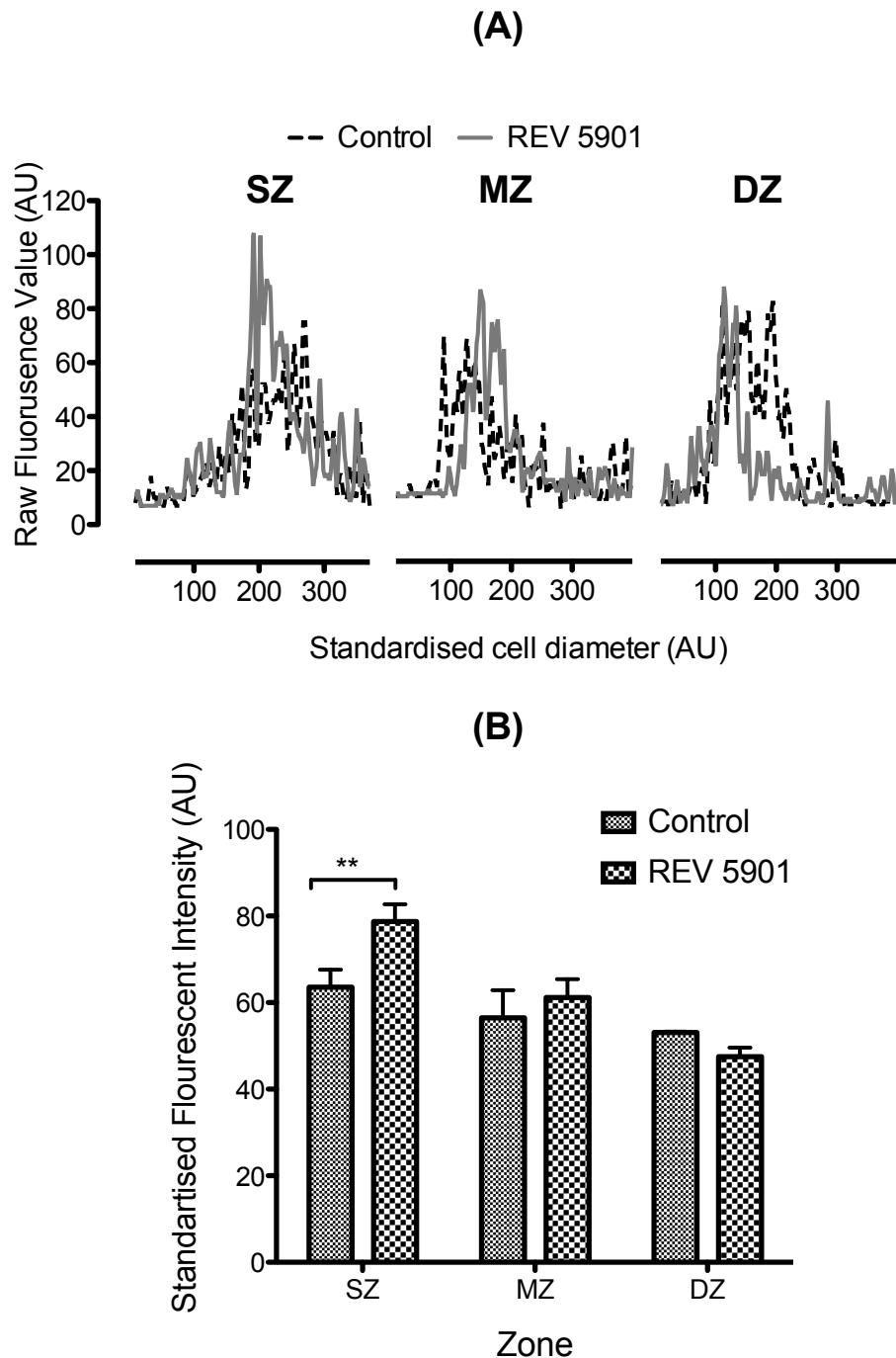
Figure 4.12 Effect of REV 5901 on F-actin organisation.

*Cartilage explants were incubated in the presence of 50 μM REV 5901 and the polymerised actin cytoskeleton labelled with Alexa 488-phalloidin. Actin was analysed by linear profiles (A) for each zone and subsequently quantified (B). Data are mean +/- SEM of N=9, n=30 cells *p<0.05, **p<0.01, ***p<0.001 all vs. control from three distinct experiments.*
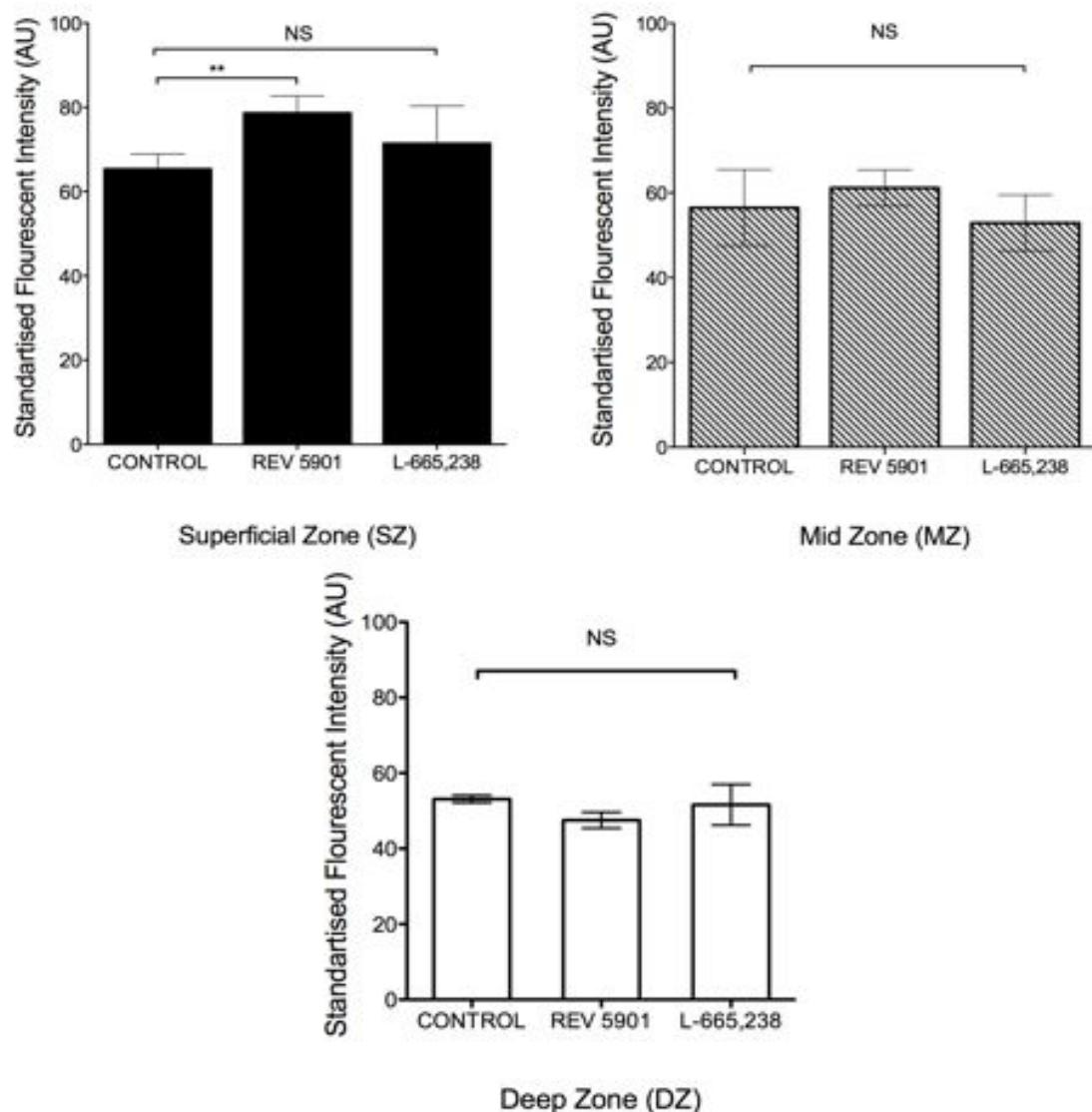
Figure 4.13 Effect of REV 5901 and L-665,238 on F-actin organisation.

*Cartilage explants were incubated 1 h in the presence of 50 μM REV 5901 or L-665,238 (REV 5901 para-isomer) and the polymerised actin cytoskeleton labelled with Alexa 488-phalloidin. Actin was analysed by linear profiles for each zone and subsequently quantified. Data are mean +/- SEM of N=9, n=30 cells *p<0.05, **p<0.01, ***p<0.001 all vs. control from three distinct experiments.*

It was observed that wortmannin induced cell death (see section 4.2.3) following single impact trauma, therefore its effects on the F-actin cytoskeleton were determined. Samples were examined by linear profiling as previously described and the F-actin organization between different zones was analysed. This method was also used to determine whether there was a potential modulation of actin depolymerisation by REV 5901 within the chondro-protective mechanisms.

Within the SZ, wortmannin significantly decreased F-actin by 30 % to 44.56 ± 8.30 AU compared to control samples 65.43 ± 3.99 AU ($p < 0.05$) and 40 % compared to REV 5901, 78.72 ± 3.99 AU ($p < 0.01$). Interestingly, the same trend, where F-actin is significantly decreased within samples incubated with wortmannin was observed for samples of the MZ where a ~25 % decrease was recorded compared to both control samples 56.49 ± 8.98 AU and 61.17 ± 4.25 AU ($p < 0.05$). There was no significant difference recorded for cells situated in the DZ for any of the experimental conditions ($p > 0.05$; Figure 4.14).

Following identification that REV 5901 chondro-protective effects were inhibited by wortmannin treatment, as well as wortmannin was reducing cell-viability post impact following treatment (Figure 4.10) the effect on F-actin organisation was determined. Interestingly, explants that had been in presence of REV 5901 exhibited increased levels of F-actin within all distinct zones but with no significant differences observed when compared to control samples alone with F-actin concentration of 68.41 ± 15.78 AU, 55.00 ± 10.75 AU or 53.34 ± 8.43 AU in SZ, MZ and DZ respectively ($p > 0.05$; Figure 4.14).

Given the effects of Latrunculin B (an inhibitor of actin polymerisation) on actin polymerization (Figure 3.8), the effect of REV 5901 in the presence of Latrunculin B (Spector *et al.,* 1989, Kerrigan and Hall, 2005) was determined on this parameter. Latrunculin B significantly reduced F-actin polymerisation for cells within all cartilage distinct zones with a 51 % reduction in SZ compared to control samples ($p < 0.001$), 45 % for MZ ($p < 0.01$) and 45 % for

DZ (p<0.01). Since REV 5901 was able to inhibit F-actin depolymerisation of wortmannin (Figure 4.14), its was investigated whether it still afforded protection in the presence of Latrunculin B in cartilage explants thus offering its protection *via* an actin regulated mechanism. Samples incubated with REV 5901 and Latrunculin B were examined and it was observed that SZ cells showed a significantly different (15 %) change compared to control samples, whilst no significant effects in the MZ or DZ compared to control zones but were observed thus showing that REV 5901 was able to inhibit the inhibitory effect of Latrunculin B on actin polymerisation (p>0.05; Figure 4.15).

Finally the effect of REV 5901 on the F-actin organisation was observed in the presence of $GdCl_3$. REV 5901 had reduced the inhibition on F-actin actin polymerisation from $GdCl_3$. F-actin organisation of the chondrocytes within the cartilage explants was restored to control levels and no significant difference was observed within the three zones (p>0.05; Figure 4.16).
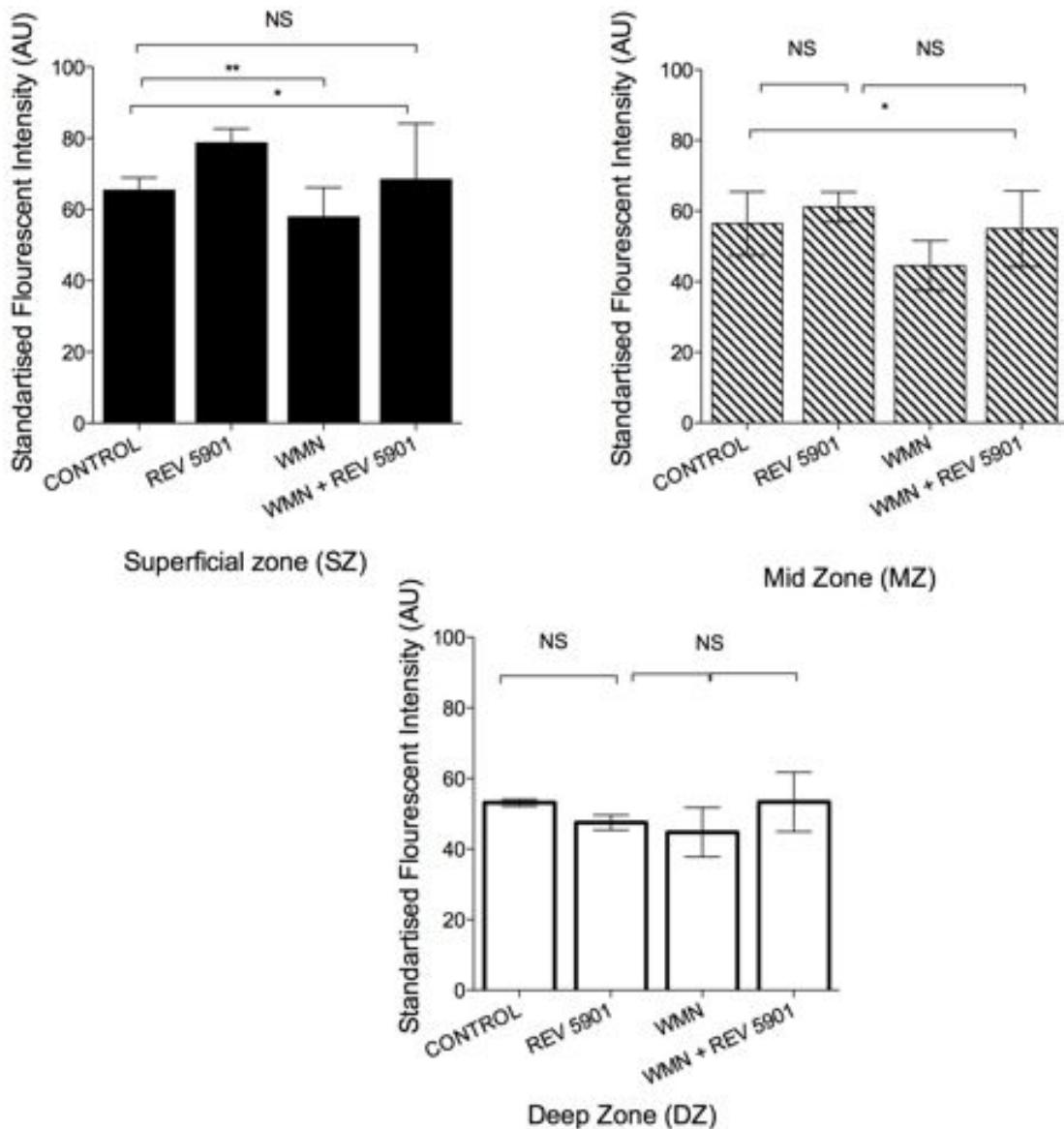
Figure 4.14 The effect of REV 5901 and wortmannin on F-actin organisation.

*Cartilage explants were incubated 1 h in the presence of 50 μM REV 5901, 10 nM wortmannin (WMN) or wortmannin (10 nM) with addition of REV 5901 (50 μM) and the polymerised actin cytoskeleton labelled with Alexa 488-phalloidin. Actin was analysed by linear profiles for each zone and subsequently quantified. Data are mean +/- SEM of N=9, n=30 cells *p<0.05, **p<0.01, ***p<0.001 all vs. control from three distinct experiments.*
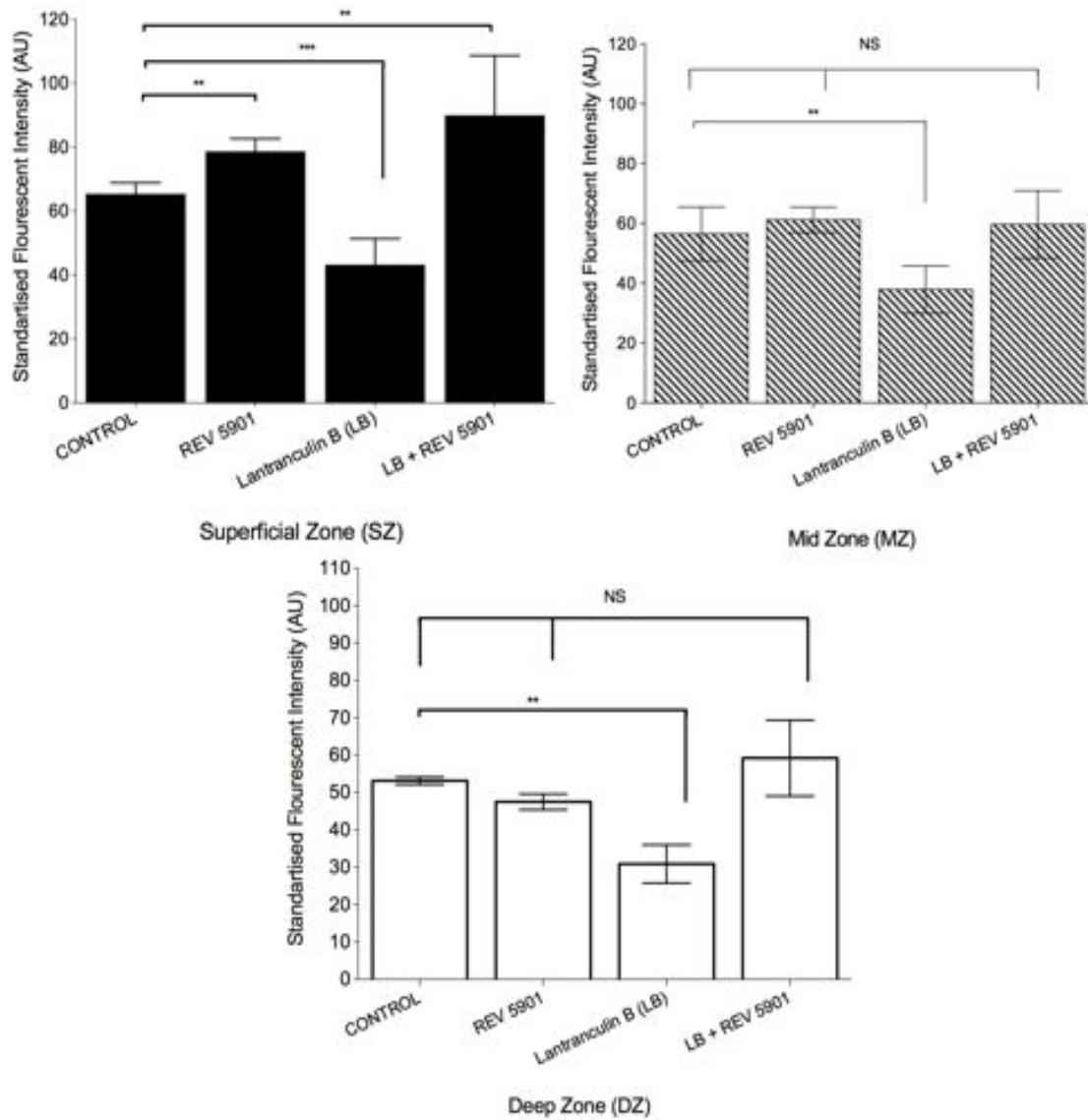
Figure 4.15 The effect of REV 5901 and *Latrunculin B* on F-actin organisation.

*Cartilage explants were incubated 1 h in the presence of 50 μM REV 5901, 10 μM Latrunculin B or Latrunculin B (10 μM) with addition of REV 5901 (50 μM) and the polymerised actin cytoskeleton labelled with Alexa 488-phalloidin. Actin was analysed by linear profiles for each zone and subsequently quantified. Data are mean +/- SEM of N=9, n=30 cells *p<0.05, **p<0.01, ***p<0.001 all vs. control from three distinct experiments.*
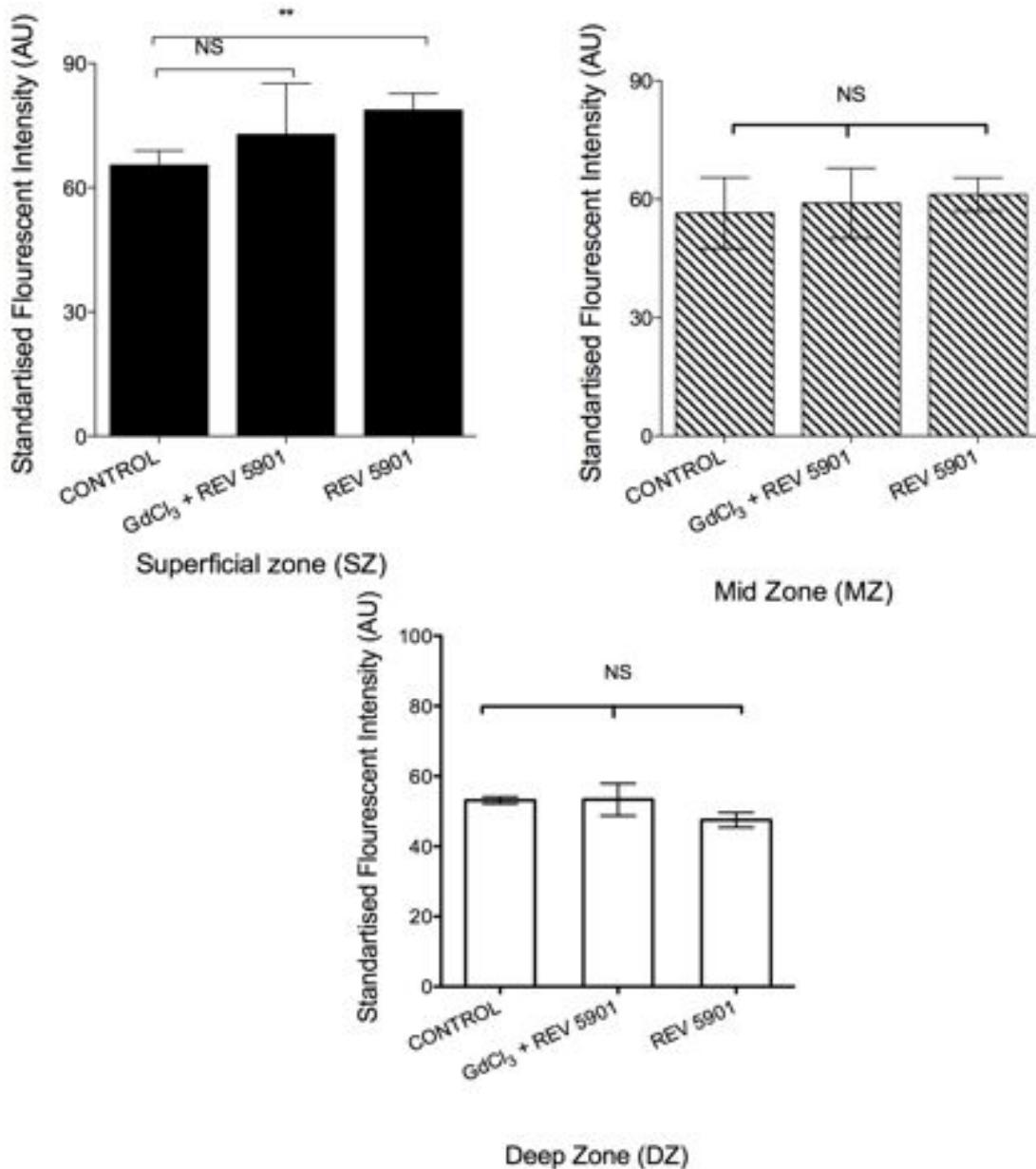
**Figure 4.16 The effect of REV 5901 and GdCl$_3$ on F-actin organisation.**

*Cartilage explants were incubated 1 h in the presence of 50 μM REV 5901, 100 μM GdCl$_3$ or GdCl$_3$ (100 μM) with addition of REV 5901 (50 μM) and the polymerised actin cytoskeleton labelled with Alexa 488-phalloidin. Actin was analysed by linear profiles for each zone and subsequently quantified. Data are mean +/- SEM of N=9, n=30 cells *p<0.05, **p<0.01, ***p<0.001 all vs. control from three distinct experiments.*

4.2.6 The effect of REV 5901 on actin binding proteins

Mechanotransduction is very important for the cell integrity and cartilage tissue and has been implicated in response to mechanical loading (Wilkins *et al.,* 2000). In chondrocytes, one of the integral functions of actin is to regulate the cell phenotype by adjustment of the cell shape and by providing the cell with mechanical integrity to resist compressive loads (Guilak, 1995, Durrant *et al.,* 1999, Blain, 2009). Therefore it was important to investigate whether different manipulations over actin organisation would lead to subsequent changes in gene expression for the associated actin proteins. REV 5901 was shown to increase actin cytoskeleton to further characterise the functions of REV 5901, RNA was extracted from freshly isolated chondrocytes that were incubated with hyperosmotic (480 mOSM) DMEM, $GdCl_3$ (100 μM) and stimulated with hyperosmotic (480 mOsm) DMEM and REV 5901 (50 μM) supplemented DMEM.

Gelsolin is a Ca^{2+} regulated protein known to sever actin filaments and actin depolymerizing factor (ADF). Densitometric analysis showed that no significant changes in gelsolin were observed following treatment with hyperosmotic medium (0.82 $\pm$ 0.07-fold) compared to control (1.01 $\pm$ 0.27-fold) expression (p>0.05). Gelsolin expression was significantly increased in chondrocytes treated with $GdCl_3$ supplemented DMEM and hyperosmotic DMEM (p>0.001) from 3.68 $\pm$ 0.95-fold compared to 1.01 $\pm$ 0.27-fold in control samples an increase ~ 3 –fold. Conversely, REV 5901 decreased the levels of gelsolin from 1.01 $\pm$ 0.01-fold in control samples to 0.72 $\pm$ 0.10- fold (p<0.001) by approximately 30 % reduction. These data indicated that REV 5901 treated cells exhibited a reduced expression of gelsolin, hence retaining a strong F-actin cytoskeleton by perhaps regulating gelsolin, with a Ca^{2+} flux (Figure 4.17).

The actin depolymerising and severing protein cofilin, is re-activated by de-phosphorylation induced by Ca^{2+} hence it was investigated in this study. Densitometric analysis (Figure 4.18) of the band intensity showed significant changes in the expression of cofilin were observed when chondrocytes were

treated with REV 5901. The levels of cofilin were reduced from 1.00 ± 0.06-fold to 0.72 ± 0.03- fold (p<0.001), by decrease of 30 %. Interestingly, when chondrocytes were incubated with $GdCl_3$ (100 μM), followed by incubation with hyperosmotic DMEM no significant difference was observed in cofilin expression with 1.15 ± 0.07-fold and 0.98 ± 0.16-fold, respectively.

Profilin has been shown to antagonise the effects of cofilin over actin (Sun *et al*., 1995). mRNA analysed from chondrocytes treated with hyperosmotic DMEM profilin expression was detected and observed to be significantly higher than chondrocytes in control samples with 2.12 ± 0.14- fold compared to 1.01 ± 0.09- fold (p<0.001) in control samples. Gd^{3+} inhibition of the SACC was observed to reduce the levels of profilin significantly to 0.68 ± 0.03- fold (p<0.001) a reduction of ~30 %. Whereas REV 5901 increased the level of profilin over control chondrocytes with 4.46 ± 0.22- fold over the control samples (p<0.001) and its effect was greater than the effect induced by hyperosmotic medium alone (Figure 4.19).

Experimental Condition	Control	HYPER	HYPER+Gd^{3+}	REV 5901
Data from Densitometry	1.00	0.82	3.68	0.72

Figure 4.17 Effect of different treatments on Gelsolin mRNA expression in chondrocytes.

Gelsolin mRNA was determined by reverse transcriptase polymerase chain reaction (RT-PCR) analysis on freshly isolated bovine articular chondrocytes treated for 1 h with hyperosmotic (480 mOsm) DMEM, REV 5901 (50 μM) and combination of GdCl$_3$ (100 μM) and hyperosmotic (480 mOsm) DMEM. A representative gel image is shown (A), along with the numeric data obtained by densitometry analysis of the gel (B). Data are mean +/- SEM of N=3 samples, *p<0.05, **p<0.01, ***p<0.001 all vs. control.
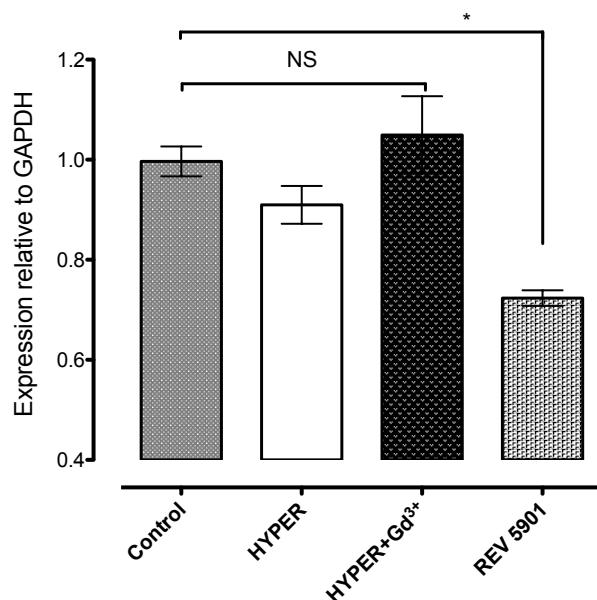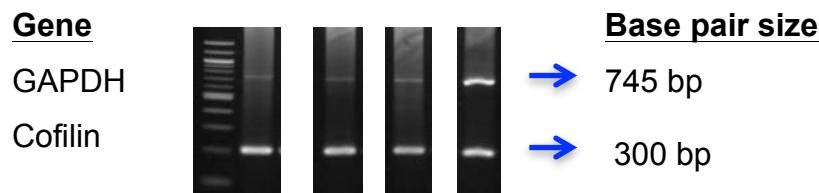
Experimental Condition Data from Densitometry	Control	HYPER	HYPER+Gd^{3+}	REV 5901
	1.00	0.91	1.05	0.72

Figure 4.18 Effect of different treatments on Cofilin mRNA expression in chondrocytes.

Cofilin mRNA was determined by reverse transcriptase polymerase chain reaction (RT-PCR) analysis on freshly isolated bovine articular chondrocytes treated for 1 h with hyperosmotic (480 mOsm) DMEM, REV 5901 (50 μM) and combination of GdCl$_3$ (100 µM) and hyperosmotic (480 mOsm) DMEM. A representative gel image is shown (A), along with the numeric data obtained by densitometry analysis of the gel (B). Data are mean +/- SEM of N=3 samples, *p<0.05, **p<0.01, ***p<0.001 all vs. control.

Figure 4.19 Effect of different treatments on Profilin mRNA expression in chondrocytes.

*Profilin mRNA was determined by reverse transcriptase polymerase chain reaction (RT-PCR) analysis on freshly isolated bovine articular chondrocytes treated for 1 h with hyperosmotic (480 mOsm) DMEM, REV 5901 (50 μM) and combination of GdCl$_3$ (100 μM) and hyperosmotic (480 mOsm) DMEM. A representative gel image is shown (A), along with the numeric data obtained by densitometry analysis of the gel (B). Data are mean +/- SEM of N=3 samples, *p<0.05, **p<0.01, ***p<0.001 all vs. control.*

4.2.7 The role of actin binding proteins in the mechanism of action of REV 5901

F-actin repetitively has been shown to be involved in cartilage chondrocyte mechanotransduction (Guilak, 1995, Grodzinsky *et al.,* 2000, Han *et al.,* 2012) influenced by mechanical loading, osmotic environment and inflammation, all of which are associated with OA (Borelli *et al.,* 1997, Goldring and Goldring, 2007, Guilak, 2011). Manipulation of osmotic pressure results in remodelling of the actin cytoskeleton with exposure to hypo-osmotic stress leading to F-actin depolymerisation and reorganization (Chao *et al.,* 2006, Blain, 2009). On the other hand, F-actin depolymerizing agents lead to reduced cell viability within *in situ* chondrocytes (Figure 3.6) therefore, it was important to investigate the role of actin binding proteins and their involvement in conveying the chondro-protective effects of REV 5901.

To access the influence of REV 5901 on the intracellular pathways of chondrocytes, eleven different conditions were compared: bovine cells before treatment (control 380 mOsm), cells treated with hyperosmotic (480 mOsm) DMEM, cells treated with REV 5901 and its para-isomer L-665,238, wortmannin, Latrunculin B, Uridine and combinations with REV 5901 including with $GdCl_3$. Protein was extracted from chondrocytes following treatment and subjected to SDS-PAGE. Membranes were then probed for cofilin, profilin and gelsolin, previously shown as F-actin modulators and related to OA (Chao *et al.,* 2006, Blain, 2009). Interestingly, only gelsolin and cofilin were detected with western blotting. Gelsolin is known to sever actin filaments thus acting as ADF and it is also known that these alterations are activated by Ca^{2+} that on the other side synergistically promote gelsolin binding to actin filaments. Densitometric analysis of the band intensity (Figure 4.20 & 4.21) revealed that REV 5901 had no significant effect over basal levels. Samples treated with REV 5901 showed exactly the same behavior as hyperosmotic (480 mOsm) DMEM and L-665,238 treated samples with 1.01 ± 0.04-fold and 0.91 ± 0.02-fold respectively therefore showing no significant difference from REV 5901 or control samples (p>0.05; Figure 4.20).

When wortmannin the PI3K inhibitor was used, expected increases in gelsolin was observed with a 1.18 ± 0.04-fold increase compared to control (p<0.05; Figure 4.21). A combination of wortmannin stimulation by REV 5901 led to a reduction in gelsolin levels compared to wortmannin alone. This reduction was not significantly different from control and REV 5901 treated samples alone, thus suggesting that REV 5901 exhibits its chondro-protective effects *via* a PI3K dependent mechanism.

Samples treated with either uridine did not show any significant differences in gelsolin levels from control samples. It was observed that samples treated with Latrunculin B significantly increased gelsolin levels with 1.11 ± 0.04-fold increase thus showing that depolymerisation of actin cytoskeleton was partly being inhibited by gelsolin (p<0.05) compared to control samples (Figure 4.21).

A

B
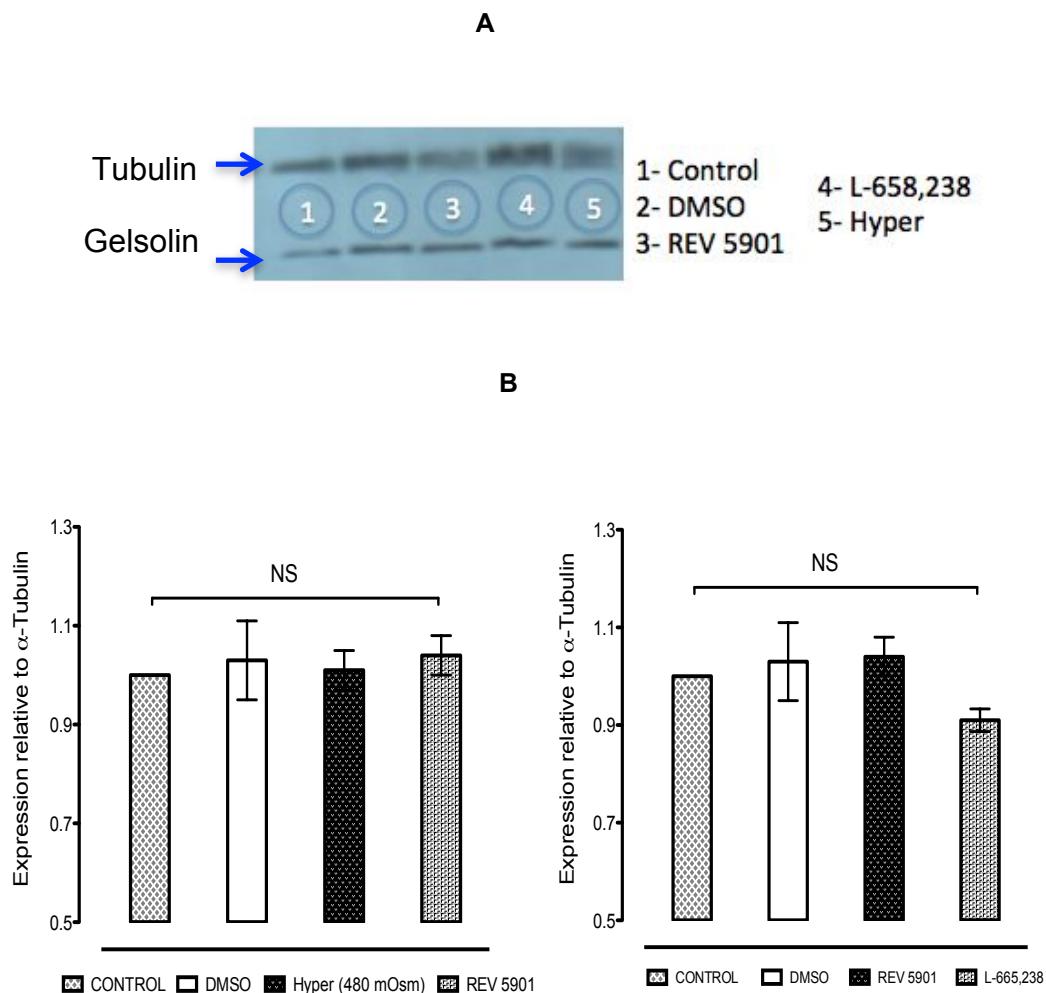
Figure 4.20 Effect of different treatments on Gelsolin protein expression in chondrocytes.

Freshly isolated bovine cells were incubated 1 h in the presence of control (isotonic 380 mOsm), hyperosmotic (480 mOsm) DMEM, DMSO (0.1 %), REV 5901 (50 μM) and L-655,238 (50 μM). Western blot analysis were utilised by standard procedures and a representative blot is shown (A), along with the numeric data obtained by densitometry analysis of the blots (B). Densitometry analysis were carried out with ImageJ® software. N=3, *p<0.05, **p<0.01, ***p<0.001 all vs. control.

A

1- Control, 2- DMSO, 3- REV5901, 4-WMN, 5- WMN+REV5901,
6-Lb, 7-REV5901+Gd^{3+}, 8-Hyper+Gd^3,

B
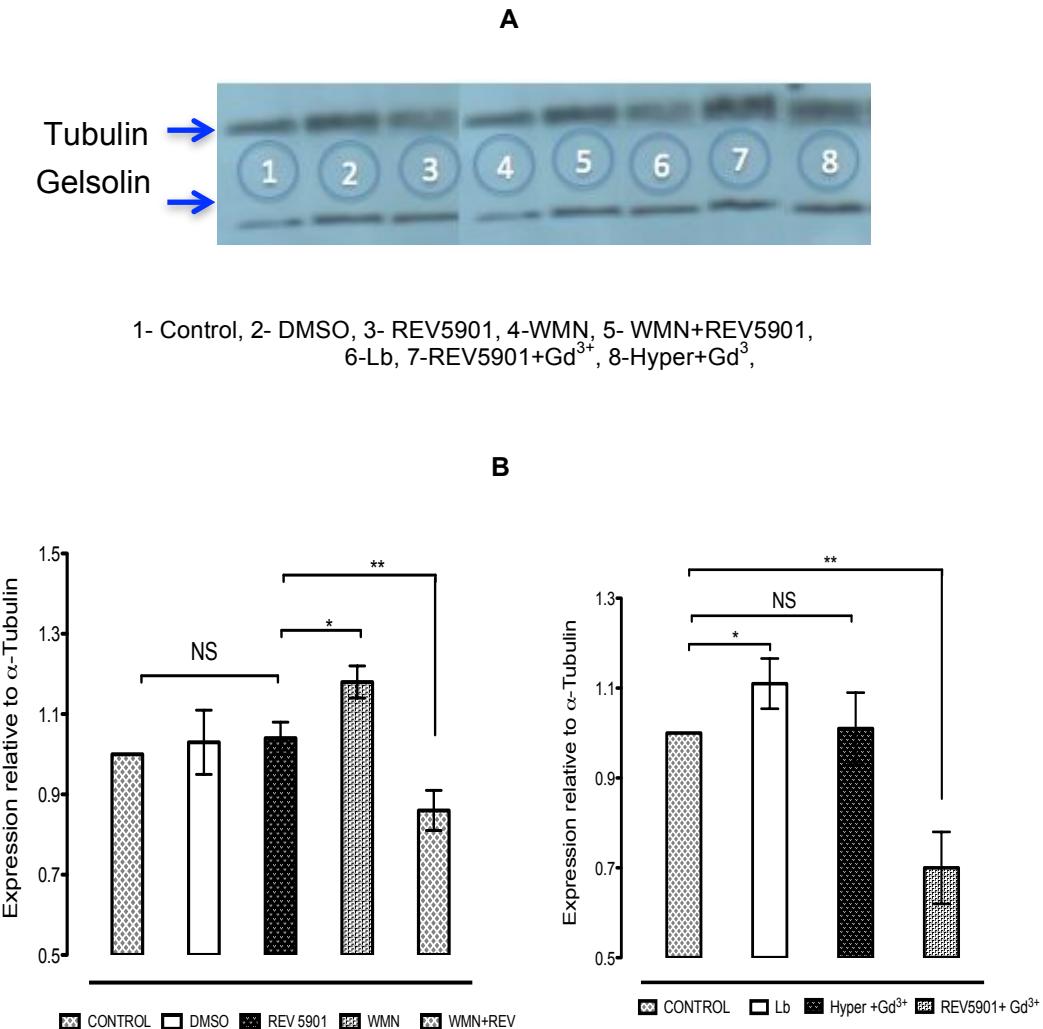
Figure 4.21 Effect of different treatments on Gelsolin protein expression in chondrocytes.

*Freshly isolated bovine cells were incubated 1 h in the presence of control (isotonic 380 mOsm), hyperosmotic (480 mOsm) DMEM, DMSO (0.1 %), REV 5901 (50 μM) and both alone and in combination with pharmacological agents. Western blot analysis were utilised by standard procedures and a representative blot is shown (A), along with the numeric data obtained by densitometry analysis of the blots (B). Densitometry analysis were carried out with ImageJ$^{®}$ software. N=3, *p<0.05, **p<0.01, ***p<0.001 all vs. control.*

Cofilin is a known actin depolymerizing protein (ADP) and together with the cofilin/ADF family they disassemble F-actin from the actin network and recycle the actin monomers (Bokoch *et al.,* 2006, Blain, 2009). Signalling through specific pathways can trigger the formation of distinct actin-dependant structures therefore, it was important to know how REV 5901 regulates the behaviour and the organisation of actin cytoskeleton.

Densitometric analysis of the band intensity (Figure 4.22) revealed that REV 5901 significantly reduced cofilin by 28 % to 0.79 ± 0.07-fold ($p < 0.05$) compared to control. Wortmannin significantly increased cofilin levels with 4.49 ± 0.08-fold ($p < 0.05$) increase compared to control, whereas treatment in combination with REV 5901 reduced expression of cofilin significantly by 65 % to 1.53 ± 0.06-fold ($p < 0.01$) over control ($p < 0.05$). L-665,238 did not significantly alter cofilin expression compared to control. Latrunculin B a known actin depolymerizing agent-increased cofilin levels by 60% to 2.48 ± 0.04-fold compared to control sample ($p < 0.01$) and by 70 % compared to REV 5901 alone ($p < 0.01$).

Interestingly, the addition of hyperosmotic (480 mOsm) DMEM did not reduce cofilin expression thus showing that the pathway behind the hyperosmotic alteration might be different from the one, which REV 5901 acts. When samples were incubated with $GdCl_3$, cofilin was significantly increased compared to control and REV 5901 samples with 1.30 ± 0.05-fold and 1.26 ± 0.04-fold ($p < 0.05$), however both are lower than that observed with Latrunculin B and wortmannin ($p < 0.01$).

A

1- Control, 2- DMSO, 3- Lb, 4-Hyper, 5- REV5901, 6-REV5901+Gd^{3+},
7- L-655,238, 8- Hyper+Gd^3, 9-WMN+ REV5901, 10 - WMN

B
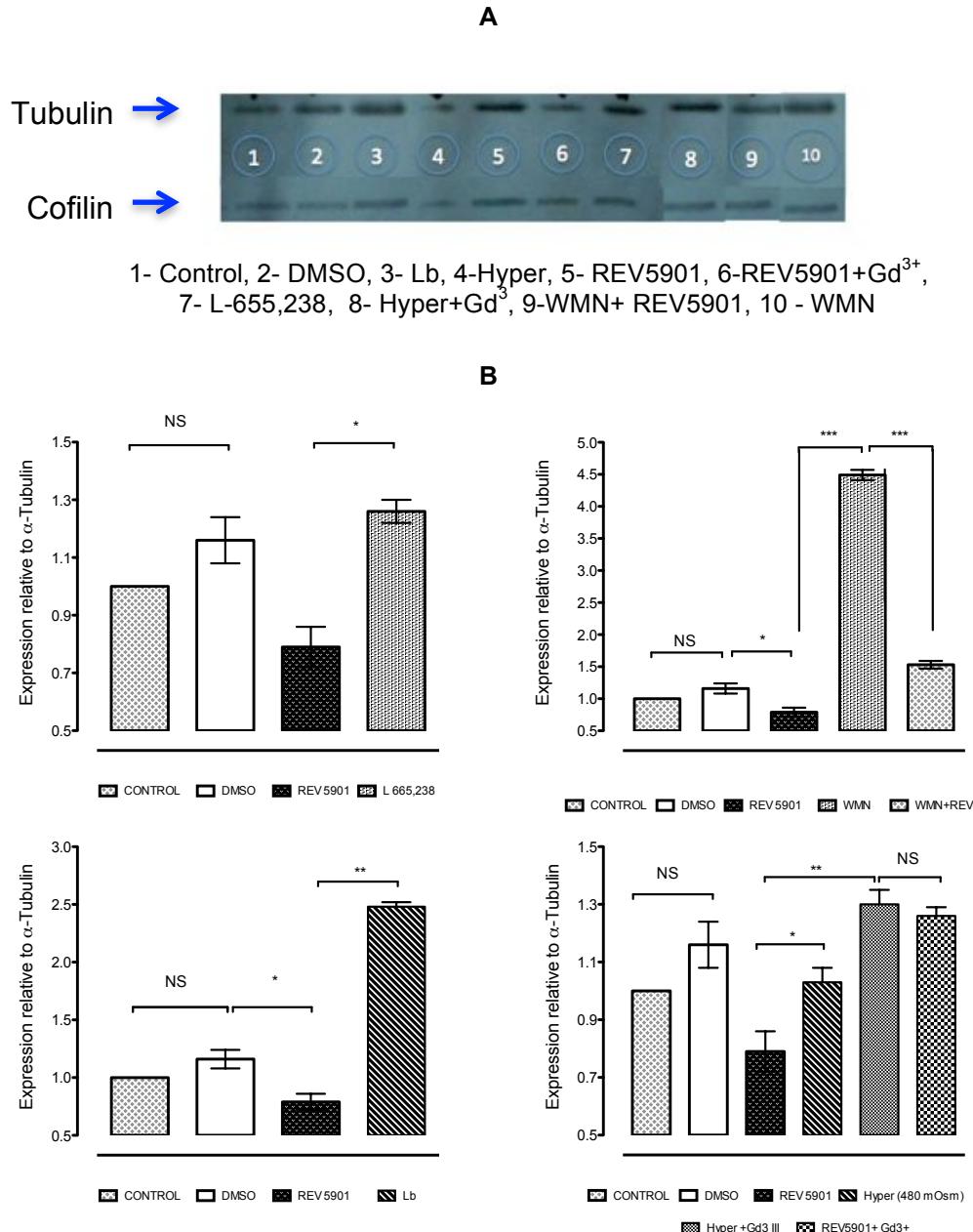
Figure 4.22 Effect of different treatments on Cofilin protein expression in chondrocytes.

*Freshly isolated bovine cells were incubated 1hr in the presence of control (Isotonic 380 mOsm) DMEM, hyperosmotic (480 mOsm) DMEM, DMSO (0.1%), REV 5901 (50 μM), L-655,238 (50 μM), Wortmannin (10 nM), Latrunculin B (10 μM), combination of $GdCl_3$ (100 μM) and hyperosmotic (480 mOsm) DMEM, $GdCl_3$ (100 μM) and REV5901 (50 μM) DMEM and Wortmannin and REV 5901 (50 μM) DMEM. A representative blot is shown (A), along with the numeric data obtained by densitometry analysis of the blots (B). Densitometry analysis were carried out with ImageJ$^{®}$ software. Data are mean +/- SEM of N=3 samples, *p<0.05, **p<0.01, ***p<0.001 all vs. control.*

4.3 Summary

Investigation of the possible chondro-protective properties of panel of pharmacological compounds revealed that REV 5901 was the most efficient at reducing cell death.

All the investigated agents were observed to decrease cell volume prior to mechanical impact by osmotically independent mechanisms supporting the observations that cell shrinkage is a chondro-protective mechanism, however once chondrocytes volume decreased to a certain level the chondro-protective effect was no longer enhanced supporting the observations in Chapter 3. Inhibition of the PI3K pathway by wortmannin increased cell death even though wortmannin reduced cell volume. When REV 5901 was used in combination with wortmannin the levels of cell death were not significantly different from control thus suggesting that the effect of REV 5901 was abolished by wortmannin suggesting that the protective effects of REV 5901 are in part *via* a PI3K dependent mechanism.

The fact that REV 5901 protected chondrocytes from impact load was surprising and appears to mimic the effects induced by hypertonic induced shrinkage. Indeed, data suggests that REV 5901 is able to induce cell shrinkage, whilst a rise in $[Ca^{2+}]_i$ is not enough to stimulate a decrease in cell volume, suggesting that the REV 5901 induced rise in $[Ca^{2+}]_i$ may not be directly involved in the associated volume change and most likely involved with the regulation of the polymerised actin cytoskeleton.

When F–actin was analysed it was observed to be increased in the superficial zone (SZ) by REV 5901 but not in the other two distinctive zones, thus showing that perhaps the difference in behaviour of the chondrocytes from different zones should be investigated. It is well documented that the actin-regulatory proteins are calcium-dependent (Erickson *et al.,* 2003, Chao *et al.,* 2006, Blain, 2009) and in chondrocytes shown that changes in $[Ca^{2+}]_i$ are involved in cytoskeletal re-organisation following osmotic challenge

(Figure 3.7) and REV 5901 incubation (Figure 4.12; Langelier *et al.,* 2000, Chao *et al.,* 2006).

To summarise, REV 5901 decreased chondrocyte volume and reduced cell death, increased F-actin in the superficial zone. It was also shown to have significant effect over the actin regulation proteins on molecular level and to be involved in the PI3K pathway. REV 5901 displayed chondro-protection and thus could be a used to develop selective compounds for novel prospective agent that can targeting short-term impact trauma hence it is important to investigate its action and regulation.

CHAPTER 5

5. DISCUSSION

Osteoarthritis (OA) is the most common form of arthritis (ARC, 2008, Loeser, 2011, Teeple *at al.,* 2013), which causes pain and disability to 8 million people within the UK (ARC, 2008) and 27 million individuals in USA (Lawrence *et al.,* 2008) it is also known to be the biggest contributor to claimants for disability living allowance in UK (ARC, 2008). Additionally, OA is the single largest indication for primary hip, knee and ankle replacement procedures in the UK recorded in 2010 with 93 %, 97 % and 72 % of the cases accordingly (National Joint Registry, 2011). Nevertheless, the burden of OA is not only in its prevalence but its economic impact is also of significant concern. Solely in the UK, it is estimated that OA brings an economic cost to the nation of approximately £5.7 billion, annually (ARC, 2008). Considering the prevalence and the significant economic, social and psychological costs of OA, it is not surprising that ongoing research into prevention of OA is an ever growing field. OA is a chronic disease with a multifactorial etiology with age being the most prominent feature (Felson and Zhang, 1998, Goldring and Goldring, 2007, Loeser, 2011). In addition, mechanically induced pathophysiological changes to the articular cartilage, subchondral bone and joint tissues can cause OA, with obesity (Felson, 1996, Iannone and Lapadula, 2010), labor and neuromuscular dysfunction (Buckwalter and Mankin, 1998, Buckwalter and Martin, 2004, Lotz and Kraus, 2010) and, high-impact sports (Felson and Andesron, 1988, Kujala *et al.,* 1995, Saxon *et al.,* 1999, Roos, 2005, Urquhart *et al.,* 2007) being implicated.

Research is gradually clarifying the response of cartilage to mechanical load and oxidative stress, be it following trauma or related to joint overloading, thereby allowing us to intercept the pathophysiological process earlier and decrease the burden of OA on society. This study has investigated the short-term cellular responses of chondrocytes to static mechanical impact and the effects of possible chondro-protective agents/pathways involved. This has allowed for a better understanding of the effects of loading on cartilage tissue, identifying valuable strategies and preventive mechanisms for individuals involved with short-term high impact or repetitive loading exercise.

The magnitude of stress measured *in vivo* in baseline physiological stress during normal walking has been calculated to be between 2 and up to 5 MPa (Urban, 1994) increasing to 18-20 MPa when rising from a chair (Hodge *et al.,* 1986). Exercise such as running or jumping contributes to physiological stresses significantly higher than the measurements recorded during walking and thus potentially can cause tissue injury. Previous studies have reported a number of models used to induce various impact forces (static or dynamic) and test parameters (weight, height and force) by utilisation of a 'drop tower' and pendulum devices. Impact heights reported in the literature range from 2.5 cm (Huser and Davis, 2007, Jeffrey and Aspden, 2007) to 50 cm (Jeffrey *et al.,* 2005) and the variation of weights used between 0.1 kg (Aspden *et al.,* 2002, Bush *et al.,* 2005) to 1 kg (Repo & Finlay, 1977, Jeffrey *et al.,* 1995, Vetramo and Sheedhom, 2007). These translate to impact forces between 1-25 N therefore, utilization of models with impact forces and delivering stress between 1-40 MPa can cause cell death and rupture(s) upon administration to articular chondrocytes and cartilage tissue (Repo and Finley, 1977, Chen *et al.,* 2001, Jeffrey *et al.,* 2005, Han *et al.,* 2012).

In this study a drop tower impact model was used (Jeffrey *et al.,* 1995, Bush *et al.,* 2005, Huser and Davis, 2007), delivering force of 1.34 N, where the chondrocyte response was investigated. The drop tower technique although a useful method of stimulating impact (Aspden *et al.,* 2002) was not used to replicate *in vivo* pressures, nor to study matrix damaging injurious loads, but to provide a sufficient force to investigate biological responses of articular cartilage and thus highlight potential areas of interest for chondro-protection. It is important to underline that within this model, the tissue does not have the protection offered by the sub-chondral bone and synovial fluid that are afforded to them *in vivo* (Bush *et al.,* 2005, Jeffrey *et al.,* 2005). There is a consiferable interest in the role of the sub-chondral bone and studies suggest (Chen *et al.,* 2003, Amin *et al.,* 2009) that the sub-chondral bone may also interact with articular cartilage via soluable mediators and influence chondrocyte survival of *in vitro* cartilage sapmles during culture. Amin *et al.,* 2009, showed that the removal of the sub-hondral bone within *in vitro* samples, results in significant increase of persantege cell death soleily within

the superfisial zone over seven days thus underlying that chondrocyte survival will be influenced during culture. To minimise any effects related to the absence of the sub-chondral bone all cartilage explants used in this study were incubated for less than 4 days.

In this study an automated cell-counting technique was used (Nedelcheva *et al.,* 2008). Using a validated and reproducible computer generated cell-counting technique to quantify cell death (Jomha *et al.,* 2003, Amin *et al.,* 2010). There is always a potential for error in such automated systems such as uneven dye loading or dye leakage, photo bleaching, unequal cell thickness, background caused by free protein or autoflorescence, etc. However, with computer based automated methods the error will be consistent between different images, therefore unlikely to effect subsequent conclusions. Lastly, the external osmolarity of *in situ* chondrocytes has not been measured directly. Cartilage tissue contains high concentration of free cations (Na^+) and low concentration of anions (Cl^-) compared to its surroundings and the precise values verified by the Gibbs-Donnan equilibrium conditions (Urban *et al.,* 1993, Urban, 1994). Thus a rise in medium osmolarity involves a corresponding rise of osmolarity in cartilage ECM that is evident from the reciprocal change observed (Figure 5.1) where by a mathematical calculation for estimating extracellular osmolarity of *in situ* volume changes have been described (Bush and Hall, 2001b).

5.1 Chondrocyte viability and mechanical injury

Blunt trauma and single injurious impact have been previously shown to compromise the mechanical properties of cartilage and reduce chondrocyte viability (Torzilli *et al.,* 1999, Bush *et al.,* 2005, Jeffrey *et al.,* 2005, Lee *et al.,* 2005, Vetramo and Sheedhom, 2007, Han *et al.,* 2012). Here the data showed that mechanical impact induced cell death of *in situ* articular chondrocytes during the first 2 minutes post mechanical injury, followed by an increasing death at the end of the observation period (30 minutes) (Figure 3.1 & 3.2). These data are in agreement with previous studies showing that chondrocyte death occurs following injurious mechanical load (Tew *et al.,*

2000, Bush and Hall, 2001b, Chen *et al.,* 2001, D'Lima *et al.,* 2001b, Bush *et al.,* 2005, Amin *et al.,* 2008) with cell death reported from as early as 3 min, and more extensive death by 25 min within the SZ (Bush *et al.,* 2005). In addition to using a drop tower (Aspden *et al.,* 2002, Bush *et al.,* 2005, Jeffrey *et al.,* 2005, Jeffrey and Aspden, 2007) to deliver the stress, scalpel injury (Amin *et al.,* 2008, Amin *et al.,* 2010), cyclic compression as well as a variety of loading rates (Ewers, 2001, Huser and Davis, 2007, Han *et al.,* 2012) have been used. So whilst mechanical load has been used to identify cell death, mechanism(s) (i.e., whether a necrotic, apoptotic or other process) are not affirmative and poorly understood.

The role of $[Ca^{2+}]_i$ in chondrocyte mechanotransduction has been studied intensively over the past few years and it would appear that the role of this prevalent intracellular secondary messenger is diverse and may depend upon numerous factors including: chondrocyte species, morphology, culture conditions and phenotype (Dascalu *et al.,* 1996, Pritchard *et al.,* 2002, Kerrigan and Hall, 2008). Previous work in bovine *in situ* chondrocytes reported that ethylene glycol tetraacetic acid (EGTA) prevented superficial chondrocyte death post-scalpel induced injury compared to calcium containing conditions (Amin *et al.,* 2009, Amin *et al.,* 2010). Therefore, to further study the mechanism of chondrocyte death post mechanical trauma $[Ca^{2+}]_o$ was removed with the addition of 2 mM EGTA, which chelates calcium ions. EGTA incubation was observed to have no significant impact over cell death in the initial stages (2 – 5 min) or at 30 min phase post mechanical trauma (Figure 3.4) conversely it was observed that chondrocyte cell volume was reduced thus exhibiting one of the universal trait of apoptosis (Bortner, 2005) indicating that perhaps initial stages of cell death post impact may be independent of calcium changes.

The data presented here suggest a role for both necrosis and apoptosis with chondrocyte death post injurious impact by necrosis likely to be the result of the blunt trauma at the point of impact and apoptosis to be spreading in the later stages ~ 30 min post loading (Tew *et al.,* 2000, D'Lima *et al.,* 2001a, Borelli, 2006, Pelling *et al.,* 2009). Similar observations have been previously

shown (Bush *et al.,* 2005), however the mechanism involved in the short-term death post mechanical impact needs to be further investigated, as only a few studies have looked at the initial cell death phases followed by observation at 2 hours post-impact. This study thus investigated a poorly understood area and suggests that initial chondrocyte death is necrotic.

5.2 Chondrocyte volume and viability in response to mechanical injury

Articular chondrocytes are constantly exposed to changing extracellular environment (Stockwell, 1971) and they need to be able to dynamically respond to variety of pressures due to mechanical loading depending on activity and loading by altering their extracellular environment (Urban, 1994). However chondrocytes, similar to many other cell types, are known to be osmotically sensitive whereby alterations in the extracellular osmolarity prompt a volume-regulation response (Bush and Hall 2001b, Amin *et al.,* 2010, Han *et al.,* 2012). It has been previously observed (Bush and Hall, 2001b) that chondrocytes behave as perfect osmometers by following the Boyle-Van't Hoff relationship and this observation was further confirmed by the data presented in this study (Figure 5.1). Both *in situ* and isolated (P_o) chondrocyte volume changes were proportional to osmotic changes thus further validating the Imaris Iso-surface volume analysis used. Furthermore, changes in relation to cell volume and ECM metabolism have been associated with the degenerative changes of cartilage that characterise OA (Buckwalter, 1977, Goldring and Macu, 2009).

Explants were exposed to a range of hyperosmotic (380 mOsm, 420 mOsm, 540 mOsm) challenges for 1 h at 37° C prior to mechanical loading to induce cell shrinkage and determine an optimal chondro-protective osmolarity. *In situ* chondrocytes were observed to decrease their volume following an increase in osmolarity resulting in an increased chondrocyte viability that reached its limitation when 540 mOsm hypertonic challenge was applied (Figure 3.3). The data reported in this study supports previous studies demonstrating that *in situ* chondrocyte shrinkage by rising medium osmolarity can protect the cell from mechanical loading and thus can act as a chondro-

protective mechanism following mechanical load (Bush *et al.,* 2005, Amin *et al.,* 2008, Amin *et al.,* 2010).
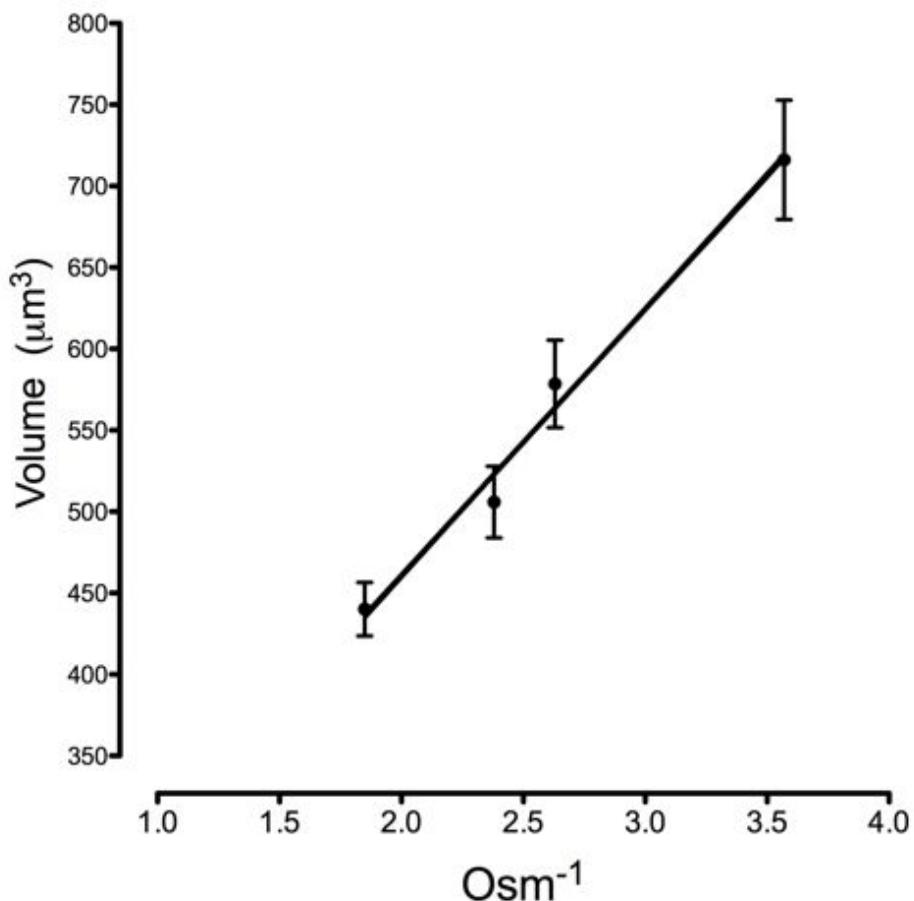
Figure 5.1 Boyle-Van't Hoff relationship in osmotically induced chondrocytes.

Chondrocyte mean volume plotted against reciprocal of the extracellular osmolarity following Boyle-Van't Hoff relationship. The y-intersept was the osmotically inactive volume b, 130 μm^3. The line of best fit has a regression coefficient (r^2=0.988) indication that in situ chondrocytes behave as perfect osmometers.

Converse to the data from this study, it has been reported that, exposure of cartilage to a high osmolarity of 600 mOsm, decreased the extent of cell death in the superficial zone (Amin *et al.,* 2010) post mechanical injury. However, the authors of the afore-mentioned study used a different model, causing mechanical injury by scalpel blade thus potentially explaining the differences observed. Interestingly, Negoro *et al*., 2008 reported that that over six days chondrocytes exposed to 570 mOsm have been shown to develop apoptosis and oncosis features (e.g. reduced cell size, apoptotic bodies fragmentation of the nucleus and blebbing) thus suggesting that increased level of osmolarity can have a negative effect on long-term cell viability. Urban *et al.,* 1993, reported that changes to cartilage hydration that prompt alterations in cell volume can influence the matrix metabolism. However, volume regulation will occur but the matrix metabolism will be determined hours later.

In many cell types an alteration in cell volume is responsible for triggering recovery mechanisms that include the activation of intracellular signaling cascades (Lang *et al.,* 2004, Lang, 2007). Over a decade ago, Erickson *et al.,* 2001, indicated that hyper osmotic stress can cause significant volume change in chondrocytes, potentially activating an intracellular second messenger signal by inducing transient increases in intracellular calcium ion $[Ca^{2+}]_i$. These observations were consistent in a number of studies (Pritchard *et al.,* 2002, Erickson *et al.,* 2003, Kerrigan *et al.,* 2008, Pritchard *et al.,* 2008) in this work we also showed that $[Ca^{2+}]_i$ is involved in changes in cell volume when hyperosmotic shock is applied (Chapter 3.2.2) and therefore, an inhibitor was used to study chondrocyte mechanotransduction *via* stretch activated ion channel (SACC). Gadolinium chloride (Gd^{3+}), is a trivalent lanthanide known to block stretch-activated calcium channels, and previously shown to inhibit $[Ca^{2+}]_i$ rise in response to hyper and hypo-tonic challenge (Erickson *et al.,* 2001, Kerrigan and Hall, 2008, Parker *at al.,* 2010), fluid flow (Yellowley *et al.,* 2000) and direct mechanical stimulation (Guliak *et al.,* 1999a). Gadolinium chloride exacerbated cell death within the presence of hyper osmotic challenge thus further implicating $[Ca^{2+}]_i$ as an important mediator for cell volume regulation.

Since volume and cell cytoskeleton have been linked in a number of studies (Guilak *et al.,* 1995, Durrant *et al.,* 1999, Kerrigan and Hall, 2005, Chao *et al.,* 2006, Sasazaki *et al.,* 2008). Research carried out by Parkkinen *et al.,* 1995, Durrant *et al.,* 1999, Erickson *et al.,* 2003, Pelling *et al.,* 2009, have examined remodelling of the cytoskeleton in response to mechanical stimuli thus the effect of cell death in response to known F-actin modulators was investigated. Latrunculin B acts as F-actin disrupting agent and Jasplankinolide acts to stabilise actin in its filamentous form. It was observed that PDC was increased by 5 % when Latrunculin B disrupted the F-actin cytoskeleton whereby Jasplankinolide PDC was decresed with 5 %, thus suggesting that F-actin plays an important role in cellular responses minutes post mechanical loading. The results presented here are consistent with previous studies where following F-actin polymerisation (Chao *et al.,* 2006, Sasazaki *et al.,* 2006).

REV 5901 inhibits chondrocyte RVD (Bush and Hall, 2001, Kerrigan and Hall, 2005, Kerrigan and Hall, 2008) and causes an increase in $[Ca^{2+}]_i$ (Qusous *et al.,* 2012). Recently it was shown that REV 5901 could induce volume changes under isotonic conditions (Qusous *et al.,* 2012). REV 5901 protected chondrocytes from impact load and appears to mimic the effects induced by hypertonic induced cell shrinkage. This would suggest that REV 5901 induced rise in $[Ca^{2+}]_i$ where by this rise can be directly involved in the associated volume change or it can be involved with the regulation of the polymerised actin cytoskeleton or both. It is well documented that the actin-regulatory proteins are calcium-dependent (Young *et al.,* 1994) and in chondrocytes it has been shown that changes in $[Ca^{2+}]_i$ are involved in cytoskeletal re-organisation following osmotic challenge (Erickson *et al.,* 2003, Pritchard *et al.,* 2008). Articular cartilage explants were incubated with wortmannin (a specific PI3K inhibitor), uridine (a mediator of intracellular calcium rise) and gadolinium chloride (Gd^{3+}), a trivalent lanthanide known to block stretch-activated calcium channels alone or in the presence of REV 5901. The PI3K pathway has been shown to play a role in cell growth, proliferation and cell survival and is also known to activate IP_3 sensitive intracellular calcium store release. Following inhibition of PI3K by wortmannin

and eliminating the effect of REV 5901 we implicated that store mediated $[Ca^{2+}]_i$ post impact plays role in the way REV 5901 acts. Since calcium is important regulator of the mechanisms of cell death and cytoskeletal reorganisation the calcium mobilising mediator uridine 5'-triphosphate was also used to further understand the mechanisms of action REV5901 exhibits. Previously it was noted that alteration in cell volume is responsible for triggering recovery mechanisms that include the activation of intracellular signaling cascades. Thus it was not unexpected that articular chondrocytes reduced their volume post impact in the presence of uridine, what was interesting was that reduced cell volume did not significantly change PDC from control samples pointing that volume regulation, changes in calcium and the addition of a third modulator such as the cell cytoskeleton must be involved in chondro-protective mechanisms.

5.3 Role and organisation of F-actin cytoskeleton within in situ chondrocytes

The lack of RVI and the hypertonic stimulation of actin polymerisation together suggest a simple yet potent pathway by which cartilage protects against joint loading and impact and specifically how this relates to the zonal structure of the tissue. Indeed, a recent study has shown that in response to compression, the SZ experiences greater changes in cell volume, height and chondron structure when compared to the other two zones suggesting that the SZ may responded differently with regards to the regulation of the actin cytoskeleton (Pritchard *et al,* 2008). As loading results in progressive fluid expression from cartilage and an increase in extracellular osmolarity (Maroudas and Bannon, 1981), this will cause decrease in chondrocyte cell-volume, a rise in $[Ca^{2+}]_i$ and depolymerisation of the actin cytoskeleton without RVI. This would confer an increase in both the stiffness and Young's modulus of the cells (Guilak *et al.,* 2002) thus causing a greater protection against loading and trauma. This assumption would therefore suggest that fissures which disrupt the collagen network that otherwise prevent the maximal hydration of the cartilage proteogylcan, will result in localised areas of lower cartilage osmolarity, changes in cellular homeostasis including

resting cell volume (Bush *et al.,* 2005) and actin depolymerisation (Pritchard *et al,* 2008). Furthermore, the progression of OA, and the loss of zones (Buckwalter, 2002) will subsequent exacerbate cartilage damage as chondrocytes in the MZ and DZ are less equipped to withstand impact force.

A key inhibitor used to study chondrocyte mechanotransduction is gadolinium chloride ($GdCl_3$), known to block stretch-activated calcium channels (Wright *et al.,* 1996) previously shown to inhibit $[Ca^{2+}]_i$ rise in response to hyper and hypo-tonic challenge (Sanchez and Wilkins, 2004, Kerigan and Hall, 2008) fluid flow (Yellowley, 2000) and direct mechanical stimulation. It is therefore conceivable that the changes in $[Ca^{2+}]_i$ observed here may relate to the regulation of the polymerised actin cytoskeleton and not directly to volume regulation. In the SZ zone there was a 30 % increase in actin polymerisation inhibited by 43% following treatment with gadolinium chloride ($GdCl_3$) and completely abrogated following the removal of extracellular calcium and the addition of EGTA. In the MZ the hypertonic challenge didn't cause increase in actin polymerisation, but F-actin was istill inhibited by 35 % using gadolinium chloride (Gd^{3+}). In the DZ, there was no change in actin concentration. These data strongly suggested a zonal specific regulation of the actin cytoskeleton with differing dependencies on intracellular calcium, whereby in the SZ, actin was regulated by stretch sensitive channels and was solely dependent upon an influx from the extracellular environment. Indeed, studies of the zone of articular cartilage have shown differences in chondrocyte gene expression (Youn *et al.,* 2006), as well as actin organisation and viscoelastic properties (Darling *et al.,* 2006).

In a similar fashion to gadolinium chloride (Gd^{3+}), wortmannin (specific inhibitor of PI3K), and Latrunculin B (an inhibitor of actin polymerisation) significantly reduced actin polymerisation within cartilage zones. Wortmannin inhibited actin polymerisation by 30 % compared to control samples and its depolymerising effect was also detected in the MZ (20 %) with no significant changes recorded for the DZ. Latrunculin B significantly reduced F-actin polymerisation by 51 % with the SZ compared to control samples and by 45 % in both MZ and DZ cells compared to control. Interestingly, REV 5901 was

not observed to block the effect of gadolinium chloride, wortmannin. Conversely, it was observed to block Latrunculin B in regards to the F-actin cytoskeleton polymerisation thus suggesting that REV 5901 can regulate actin polymerisation by a number of mechanisms. To investigate the mechanism involved and to understand the effects on actin polymerization the changes in expression of F-actin calcium regulated proteins were observed.

5.4 Changes in expression of F-actin, calcium regulated proteins in the mechanism of action of REV 5901

The expression of F-actin regulated proteins have not been investigated in depth and are yet to be elucidated. In this study we observed their function in relation to REV 5901 and its mechanism of action. It is important to note that the data recorded is at a preliminary stage and a more extensive investigation should be carried out before the data is conclusive. The role of three F-actin regulated proteins was investigated: cofilin, an ubiquitous actin binding factor that controls actin polymerisation and reorganisation in eukaryotes (DesMarais, *et al.,* 2005, Huang *et al.,* 2006), gelsolin, a major actin capping filament (Song *et al.,* 2006) and profilin, an actin monomer binding protein linked to dynamic rearrangements of actin cytoskeleton and also shown to antagonise the effect of cofilin (Sun *et al*., 1995, Bottcher, *et al.,* 2009)

Multiple signalling pathways have been proposed in mechanisms for regulation of cofilin, gelsolin and profilin including Ca^{2+}, cAMP and PI3K (Huang *et al.,* 2006, Bottcher, *et al.,* 2009). In addition, profilin and gelsolin have been shown to bind phosphatidylinositol 4,5-bisphosphate [PIP2] (Burtnick *et al.,* 2004). For the purpose of this study we compared mRNA levels of gelsolin, cofilin and profilin and recorded that REV 5901 and increased hyperosmolarity showed reduced levels of gelsolin in comparison to gadolinium chloride, thus further implication of the role of the SACC in regulation of actin dynamics and cell shape regulation. Cofilin gene

expression levels were significantly reduced by REV 5901 and a significant increase in profilin (cofilin antagonist) was recorded. It is interesting to note that profilin levels were significantly increased in cells treated with hyperosmotic DMEM but cofilin expression was not significantly different compared to cells incubated in control DMEM. Data suggests that volume regulation and Ca^{2+} influx might be related to profilin regulation, with REV 5901 managing to reduce cofilin *via* an independent mechanism. Perhaps involving another Ca^{2+} regulated pathway such as protein kinase C (PKC).

To access the influence of REV 5901 on intracellular pathways, proteins were extracted and membranes probed, and only gelsolin and cofilin were detected. Gelsolin is a major actin capping filament known for capping and severing proteins and has been reported to be regulated by PLC activity (Song *et al.,* 2006) and was not significantly altered by REV 5901, L-665,238, hyperosmotic DMEM and uridine. Cells pre treated with the PI3K inhibitor wortmannin or Latrunculin B (an inhibitor of actin polymerisation) exhibited an increase in gelsolin, therefore proposing a potential mechanism for why F-actin levels were decreased, when zone actin cytoskeleton organisation was examined.

Cofilin (actin depolymerising protein) levels were significantly reduced by addition of REV 5901 by 28 % (data is in reaffirmation of mRNA analysis) and significantly increased by addition of Latrunculin B by ~ 3-fold (compared to REV 5901 alone) and wortmannin by ~3.2-fold. This data confirms previous studies that looked at cofilin in relation to Latrunculin B (Chiu *et al.,* 2010) and wortmannin, which has previously been shown to activate cofilin by a number of stimuli (Bramburg and Bernstein, 2010). Interestingly, levels of cofilin were substantially increased in cells treated with L-655,238 (REV 5901 para-isomer) suggesting a potential reason why the chondro-protective effect over time was reduced for cells treated with L-655,238 and why REV 5901 was the most effect para-isomer when compared together.

Further investigation into the actin cytoskeleton needs to be carried out and data for vimentin and tubulin needs to be investigated before a final

conclusion can be made, however the current data suggests that REV 5901 works via PI3K (Figure 5.2).
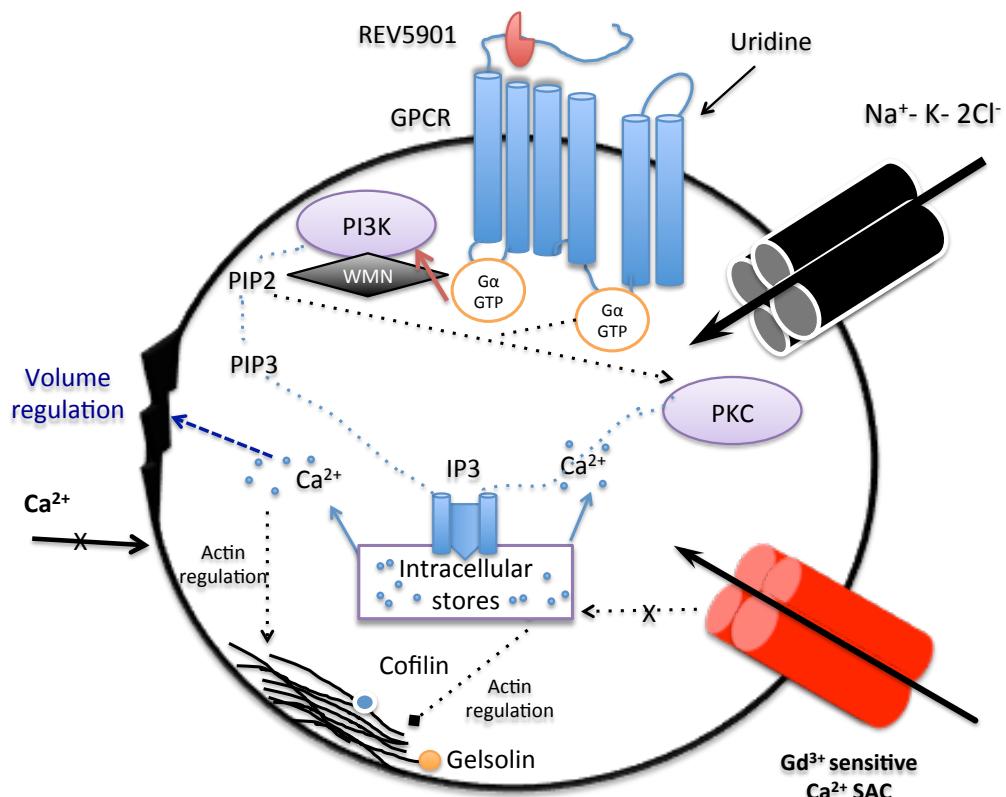
Figure 5.2 Theoretical Model of the mechanism of REV 5901.

In order to investigate the chondro-protective action of REV 5901 shown in this study a theoretical model of its mechanism is presented here. REV 5901 is known to increase Ca^{2+} and Ca^{2+} has been reported as a very important factor in mechanotransduction, volume regulation and cell death. By blocking the Ca^{2+} rise with wortmannin (WMN) a blocker of the PI3K pathway and by $GdCl_3$ inhibitor of the SACC the chondro-protection offered by REV 5901 was inhibited. In addition, this triggered changes in F-actin thus also implicating cell cytockeleton is a major factor of interest in chondro-protection.

5.5 Conclusions

In conclusion, the results presented here demonstrate the mechanism of chondro-protection by hypertonic challenge and REV 5901 and suggest a mechanism by which cell shrinkage and the lack of RVI can protect chondrocytes from impact trauma.

The regulation of the chondrocyte actin cytoskeleton remains to be elucidated, but it would appear to be dependent upon both an increase in intracellular calcium and stretch. Future work is required to determine the mechanism of the calcium induced actin polymerisation and the regulation of cell volume, which when fully elucidated, may offer a potential mechanism to protect chondrocytes from impact trauma, as well as offer an insight into the progression of OA.

5.6 Future Work

To enhance these data, future research into the forms and mechanisms of cell death involved needs to be applied. Methods including TUNEL assay and Annexin V binding for apoptosis and LDH content for necrosis will be utilised on post impacted articular cartilage explants to rule out or confirm whether apoptotic or necrotic cell death is involved and offer a quantification of data. Additional PCR and Western blots will be performed to determine changes in expression of cofilin, phospho-cofilin, profilin and gelsolin post impact thus furthering the investigation of the mechanism behind cell death.

Having established that the cytoskeleton plays an important role in cell biomechanics, further investigation into the live cytoskeletal dynamics following impact of *in situ* chondrocytes and determination of real time changes following both osmotic and impact stimuli can be performed. This would involve the utilisation of CLSM techniques using a high-speed 4D imaging systems and transfection of live cells with key proteins such as actin (Figure 5.3), tubulin and vimentin, these would be fluorescently labelled to investigate the responses and their remodelling to impact and

pharmacological agents in real-time. Once cytoskeleton analysis has been carried out, a novel method of analysing the cytoskeleton can be developed called 'Filament Tracer Analysis,' a feature of Imaris 7.1 software which can be used for offering a better understanding of this key element for cell survival (Figure 5.4). The outcome of the analysis may aid studies in actin-dependent signalling pathways, including responses to cytokines, growth factors and mechanotransduction pathways.

Subsequently, a human *in situ* chondrocyte model needs to be developed, where by investigation into the real time cytoskeletal dynamics would be valuable to integrate physiological, mechanical, and biochemical stimuli. Thus helping tissue engineering in the creation of an engineered tissue with sufficient extracellular matrix (ECM) composition, geometry, and material properties before *in vivo* implantation. Since tissue growth is complex and regulated by numerous factors such as nutrients, mechanical loading and growth factors, different bioreactors such as continuous fluid flow (Gemmiti and Guldberg, 2006) and concentric cylinders (Wick and Farooque, 2009) have been used. Loading cartilage *in situ* results in the generation of various complex stimuli (Chapters 3 & 4) that modulate chondrocytes activities and a better understanding of loading of articular cartilage and the effect on cytoskeletal dynamic will be inevitable for large scale tissue production bioprocess.

Further studies are needed to identify biological and mechanical mechanisms related to alterations stimulated by load. Moreover, the expression of proteins and genes to loading should be examined on a tissue level response. Present studies (Poulet *et al.,* 2011, Ko *et al.,* 2013) have looked into development of *in vivo* mouse models where further investigation of the articular cartilage integrity to high impact spots exercise was measured. Therefore potential investigation of REV 5901 in these *in vivo* models could provide additional information to the suitability of this compound as a chondro-protective agent prior to human based studies.
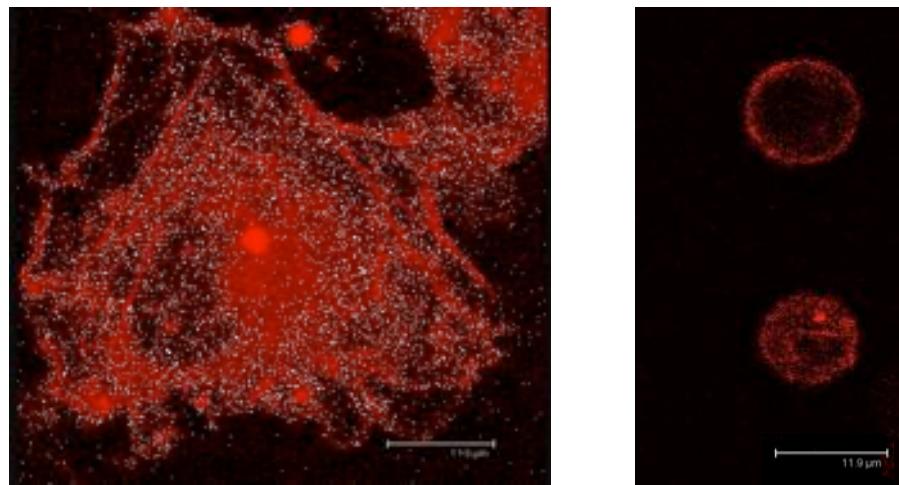
Figure 5.3 Transfected live cells with Red Fluorescent Actin.

Preliminary work on live cell transfected with CellLight® RFP Actin was carried out to establish a protocol for future work (A) chondrocyte cell line C-20/A4 and (B) bovine primary chondrocytes (Po). Images were acquired with CLSM Leica SP2 and the appropriate TRITC filters in a temperature-controlled environment, cells were imaged with x63 immersion objective, 4x zoom. Bar = 11.9 µm
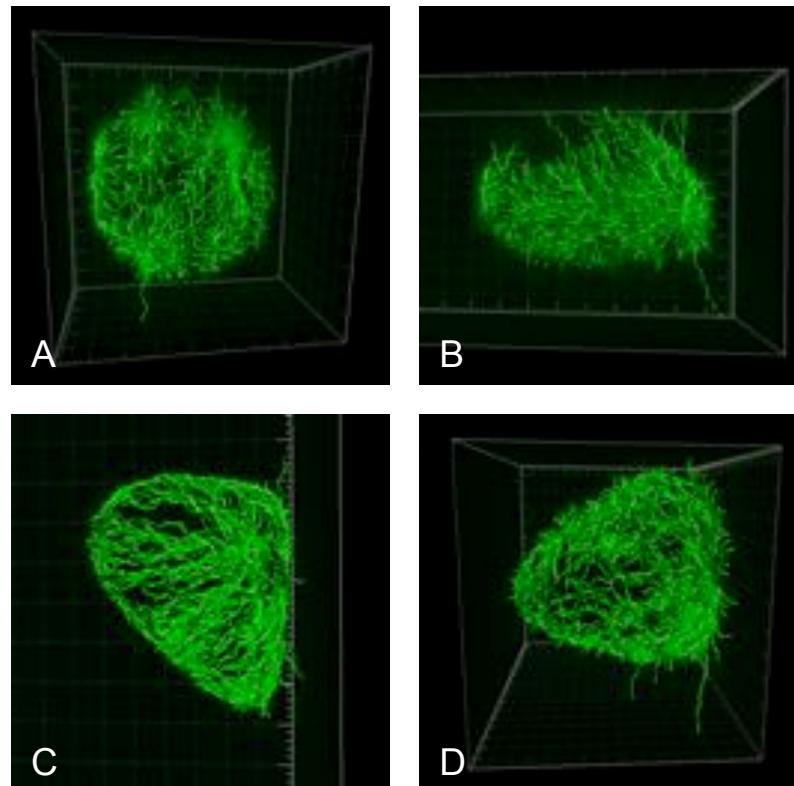
Figure 5.4 Novel method for cytoskeletal analysis.

To investigate the behaviour of cytoskeleton dynamics a novel method that uses filament tracer feature of Imaris 7.1 can be developed where filament traser feature is applied to phalloidin labled cells to distinguish F-actin filaments in various conditions. (A) SZ cell in control (280 mOsm) DMEM (B) SZ cell in hypertonic (380 mOSm) DMEM (C) DZ cell in control (280 mOsm) DMEM and (D) DZ cell in hypertonic (380 mOSM) DMEM.

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Appendices

Appendix I Conference Abstracts

The role of the actin cytoskeleton in chondrocyte protection following single impact.

Yanitsa Nedelcheva, Stephen Getting, Mark Kerrigan

School of Life Sciences, University of Westminster, London, United Kingdom.

Impact trauma results in cartilage damage and a pre-disposition to osteoarthritis, which leads to joint pain, stiffness and ultimately failure. Chondrocytes, the sole resident cell-type in cartilage, are responsible for the maintenance of the extracellular matrix (ECM) in response to their physico-chemical environment. As chondrocytes do not divide, it is therefore essential to find a method to protect chondrocytes from impact and thus maintain chondrocyte viability and cartilage integrity.

Full-depth cartilage was removed from bovine metacarpal-pharengeal joints (♀; 18-24months of age) under aseptic conditions. Explants were incubated with 5µM calcein-AM and 1µM propidium iodide for 30mins and impact experiments performed by drop-tower with a single impact of 0.07, 0.131 & 0.263J. The effect was studied by confocal laser scanning microscopy (CLSM) and quantified using an automated method with Imaris 'Spots'. The effect of cell shrinkage and stretch-sensitive calcium channels were determined using hypertonic media (380mOsm) and 100µM $GdCl_3$ respectively. Actin organisation was measured on Alexa-488 phalloidin labelled chondrocytes by CLSM. Cytokines Interleukin-1 Beta (IL-1β), and Monocyte Chemoattractant Protein 1 (MCP 1) levels were determined in supernatants by commercially available ELISA and nitric oxide (NO) by greiss assay. All data are expressed as Mean ± s.e.m. *$p < 0.05$ vs. control, n = 4-6 at three determinations.

Data were analysed using Imaris 6.02 'Spots' and following 20mins post impact, there was a significant decrease in cell viability ($p < 0.05$) for both higher impact forces when compared toinitial cell death and the lower impact force of 0.07J. These data compared to previously published results thus validating this technique (Bush *et al.,* 2005). A pre-incubation in hypertonic media protected chondrocytes from cell death whereby at 20mins death was decreased from 9.22 ± 1.65% to 3.42 ± 0.60 % ($p < 0.01$), inhibited by 100µm $GdCl_3$ (20.45 ± 3.88% death at 20mins) thus implicating stretch-sensitive calcium channels. The polymerised actin cytoskeleton was labelled using Alexa 488-phalldoidin. Hypertonicity increased cortical actin by 29.1 ± 0.13% for all zones of cartilage ($p < 0.01$) and was inhibited by 100µm GdCl3. Analysis of the supernatants revealed an increase in IL-1β (1.5±0.15pg/g), (MCP1 523±126 pg/g), NO (1241±173µg/ml) over the 24hour period post impact $p < 0.05$ vs control values.

These data show a new method for the analysis of impact-mediated chondrocyte viability and that hypertonic chondro-protection is mediated by a stretch-sensitive, calcium-dependent polymerisation of the actin cytoskeleton. Cartilage impact caused the release of pro-inflammatory cytokines that will decrease cartilage viability. Therefore, therapeutic modulation of actin polymerisation will protect chondrocytes from impact damage and could offer a suitable method to decrease the pre-disposition to osteoarthritis.

Bush, P., *et al.,* Osteoarthritis and Cartilage, 2005. 13(1): p. 54-65

REV 5901: the protective effect on bovine articular chondrocytes following single impact trauma.

<u>Y. Nedelcheva</u>, S. J. Getting, M. J. Kerrigan

School of Life Sciences, University of Westminster, London, United Kingdom.

A single impact load to a diarthrodial joint can cause permanent damage to the cartilage extracellular matrix (ECM), including surface fissures, loss of proteoglycan, cell death and a subsequent pre-disposition to osteoarthritis (Bush *et al*., 2005, Scott & Athanasiou, 2006). Chondrocytes being the sole resident cell-type in cartilage are responsible for the maintenance of the ECM therefore, as these cells do not divide it is vital to find a mechanism that protects chondrocytes from the effects of impact and thus prolongs their viability.

Full-depth cartilage was excised from bovine metacarpal-phalangeal joints (18-24 months of age; obtained from the local abattoir) under aseptic conditions into DMEM. Explants were incubated with 5µM calcein-AM and 1µM propidium iodide for 30 min and impact experiments performed by drop-tower with a single impact of 0.131J. Samples were incubated alone or in the presence of REV5901 (50µM, 30mins). Cell viability was determined at 0, 5, 10, 20 & 30mins post impact by confocal laser scanning microscopy (CLSM) and quantified using an automated method with Imaris 7.0 'Spots' as previously described (Nedelcheva *et al*. 2008). Actin organisation was measured using Alexa-488 phalloidin labelled chondrocytes by CLSM, whilst RT-PCR evaluated alterations in cofilin and profilin mRNA levels. Amplification products were visualized by ethidium bromide fluorescence on 2% agarose gels and changes in expression quantified. All data are expressed as Mean ± s.e.m. *P<0.05 vs. control, n = 4 determination in triplicate.

Impact led to a decrease in chondrocyte viability of 11.23 ± 1.24% at 30mins, this was significantly reduced in chondrocytes pre-treated with REV5901 and caused a reduction in cell death by 51% to 5.44%± 0.59% (p<0.001; Student's t-test). REV5901 caused an increase in cortical actin staining by 20% to 78.72 ±3.99 AU within the superficial zone of articular cartilage (P<0.01; Student's t-test) with no significant alterations observed in the mid and deep zones with values of 61.17 ± 4.25 AU and 47.50 ± 2.12 AU respectively. When comparing the differences between the mid and deep zone no significant difference was observed (P>0.05). RT-PCR of mRNA extracted from REV5901 treated chondrocytes indicated alterations in cofilin and profilin mRNA compared to untreated chondrocytes. There was no significant difference detected in gelsolin mRNA.

These data highlight that REV5901 protects chondrocytes from acute trauma due in part to chondro-protective polymerisation of the actin cytoskeleton. Therapeutic modulation of the actin modulation and the pathway that REV5901 is involved in could offer novel therapeutic opportunities for

prevention of irreversible cartilage damage from acute impact trauma and minimize pre-disposition to osteoarthritis.

Bush, P., *et al*., (2005). Viability and volume of *in situ* bovine articular chondrocytes—changes following a single impact and effects of medium osmolarity. *Osteoarthritis Cartilage,* 13(1), 54-65

Nedelcheva, Y., *et al*., (2008). The role of the actin cytoskeleton in chondrocyte protection following single impact. *E-journal of the British Pharmacological Society.* 148P

Scott, C., Athanasiou, K., (2006). Mechanical impact and articular cartilage*. Critical reviews in biomedical engineering.* 34(5), 347-378

REV5901: an investigation on the chondroprotective effect in bovine chondrocytes following single impact trauma.

Y. Nedelcheva, S. J. Getting, M. J. Kerrigan

School of Life Sciences, University of Westminster, London, United Kingdom.

Chondrocytes, the sole resident cell-type in cartilage are responsible for the maintenance of the extracellular matrix and as they do not divide it is vital to find a protective mechanism against impact trauma. A single impact load to a joint causes subchondral bone fracture resulting in permanent cartilage damage to the ECM, including surface fissures, loss of proteoglycan, cell death and pre-disposition to osteoarthritis (Bush *et al.,* 2005, Scott & Athanasiou, 2006).

Full-depth cartilage was excised from bovine metacarpal-phalangeal joints (18-24 months of age, obtained with permission from a local abattoir) under aseptic conditions. Explants were incubated with 5µM calcein-AM and 1µM propidium iodide for 30 min and impact experiments were performed by drop-tower with a single impact of 0.131J. Samples were incubated alone or in the presence of REV 5901 (50 µM, 30 min). To determine the mechanism of REV 5901 we used Wortmannin (10nM) and Uridine (100µM). Cell viability was determined time points of 0, 5, 10, 20 & 30 mins post impact by confocal laser scanning microscopy (CLSM) and quantified using an automated method with Imaris 'Spots'. Data for cell volume was determined in non-impacted samples and 30 min post impact. Western blot analysis were used to determine alterations in actin binding proteins: cofilin, gelsolin, profilin, and phospho cofilin to. Protein bands were analysed by Image J software and changes in expression quantified. All data are expressed as Mean ± s.e.m. *$P<0.05$ vs. control, n = 4 determination in triplicate.

Impact led to cell death which was significantly reduced by a pre-incubation with REV 5901 from 10.92±1.02% to 5.44%±0.59% ($p<0.001$). Samples treated with Uridine caused a reduction of cell death that was significantly lower from the control samples 8.10±0.40% ($p<0.05$) but couldn't reach the chondro-protective effect offered by REV 5901 ($p<0.01$). In contrast, samples treated with Wortmannin increased cell death to 12.02±1.83%. Interestingly, cell death was significantly reduced in samples treated in combination with Wortmanin and REV 5901 8.58±0.54%. Data for volume changes showed significant decrease in volume for cell treated with Wortmannin 359.9±17.67µm^3, compared to 716.136±37.81µm^3 and 552.68±27.26 µm^3 for control ($p<0.001$) and REV 5901 ($p<0.001$) respectively. Nevertheless, cell volume for cells treated with Uridine significantly decreased in comparison to the control 608.3±24.32µm^3 ($p<0.05$). In addition, when cells were treated with a combination of Wortmannin and REV5901 cell volume size was restored to 538.09±30.6µm^3. Western blot analysis showed that only cofilin and gelsolin from the actin binding proteins were expressed in all the samples, no profilin or phospho-cofilin were detected.

These data confirm the therapeutic opportunities offered by REV 5901 and it's chondro-protective properties in part due to the polymerisation of the actin cytoskeleton. We suggest that the pathway that is involved is via the phosphoinositide 3-kinases (PI3Ks) and could offer novel therapeutic opportunities for prevention of irreversible cartilage damage from acute impact trauma.

Bush, P, *et al.,* (2005). Viability and volume of *in situ* bovine articular chondrocytes—changes following a single impact and effects of medium osmolarity. *Osteoarthritis Cartilage,* 13(1), 54-65

Scott, C, Athanasiou, K (2006). Mechanical impact and articular cartilage. *Critical reviews in biomedical engineering.* 34(5), 347-378

Appendix II List of Publications

Nedelcheva, Yanitsa and Getting, Stephen J. and Kerrigan, Mark J.P. (2008). *The role of the actin cytoskeleton in chondrocyte protection following single impact.* pA2 Online. From the University of Brighton, Winter 2008 Meeting: Proceedings of the British Pharmacological Society, 6(4).

Nedelcheva, Yanitsa and Getting, Stephen J. and Kerrigan, Mark J.P. (2010). *REV5901: the protective effect on bovine articular chondrocytes following single impact trauma.* In: Physiological Society Meeting 19, University of Manchester.

Nedelcheva, Yanitsa and Getting, Stephen J. and Kerrigan, Mark J.P. (2010). *REV5901: an investigation on the chondroprotective effect in bovine chondrocytes following single impact trauma.* In: Physiological Society Meeting 21, University of Durham.

Appendix III - Buffers

TBST Buffer

1. 10 ml 1M Tris-HCl pH 8.0
2. 30 ml 5M NaCl
3. 500 µl Tween-20
4. ddH$_2$O up to 1 l

Hot Lysis Buffer

1. 0.5 M NaCl
2. 5 mM EDTA
3. 1% Triton X100
4. 10 mM Tris-HCl pH 7.4
5. 5 M DTT