BIOLOGICAL EFFECTS OF LOW FREQUENCY ULTRASOUND ON BONE AND TOOTH CELLS

by

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Abstract

Little is known about the biological effects of ultrasound on dental-derived cells and whether ultrasound may be used as a therapeutic tool in dental care. This thesis has investigated the functional responses of *in vitro* osteoblast and odontoblast model cell lines to low frequency ultrasound as a potential tool for dental tissue repair. Two methods for ultrasound delivery were used to stimulate cells *in vitro*; a dental ultrasonic scaler (EMS) capable of emitting ultrasound at a frequency of 30kHz; and the DuoSon (SRA developments) therapeutic ultrasound exposure system, which allowed the comparison of kHz, MHz and a combined frequency ultrasound. Odontoblast-like cells positively responded to all ultrasound frequencies applied and can increase VEGF expression, increase cell number and increase mineral deposition by enhancing differentiation when compared with sham-treated control. Furthermore, enhanced wound healing by increased cell migration and cell proliferation was demonstrated in ultrasound-stimulated osteoblast-like cells. Ultrasound induced a dose-dependent response in β-catenin staining in both odontoblast and osteoblast model cell lines, which implicates the Wnt/β-catenin pathway as a possible mechanism for intracellular ultrasound transduction. Taken together, it is tempting to speculate that direct low frequency ultrasound stimulation of the dentine-pulp complex or alveolar bone may be able to initiate or enhance regenerative events.

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10% DMEM: Standard cell culture medium

β-catenin: Beta-catenin

β-GP: Beta-glycerophosphate disodium salt hydrate

β-ME: Beta-mercaptoethanol

ALP: Alkaline phosphatase

AA: Ascorbic acid

BMP: Bone morphogenic protein

BMU: Basic multicellular unit

BSA: Bovine Serum Albumin

BSP: Bone sialoprotein

Cbfa1: Core bonding factor a 1

DAB: Diaminobenzidine

Dex: Dexamethasone

Dvl: Dishevelled

DMEM: Dulbecco's Modified Eagle Medium

DMP: Dentin matrix protein

DMSO: Dimethyl Sulfoxide

DPP: Dentin phosphoprotein

DSP: Dentin sialoprotein

DSPP: Dentin sialophosphoprotein

ECM: Extracellular matrix

EDTA: Ethylenediaminetetraacetic acid

FBS: Fetal Bovine Serum

FGF: Fibroblast growth factor

FZ: Frizzled

GAGs: Glycosaminoglycans

GAPDH: Glyceraldehydes-3-phosphate-dehydrogenase

GSK: Glycogen synthase kinase

H: Hertz

HF: High frequency

HSP: Heat shock protein

HIFU: High Intensity Focused Ultrasound

IGF: Insulin-like growth factor

IGF-2: Insulin-like growth factor-2

IMS: Industrial Methylated Spirits

Immuno: Immunocytochemistry

K2HPO4: Dipotassium phosphate

KH2PO4: Monopotassium phosphate

kHz: Kilohertz

LF: Low frequency

LIPUS: Low Intensity Pulsed Ultrasound

LRP: LDL receptor related protein

LEF1: Lymphoid enhancer factor-1

MAPK: Mitogen-activated protein kinase

MC3T3-E1: Murine-derived clonal cells-E1

MDPC-23: Mouse-derived dental pulp cells-23

MHz: Megahertz

MPa: Megapascals

mW: Milliwatt

mW/cm^2: Milliwatt per centimetre squared

NaCl: Sodium Chloride

NCP: Non-collagenous proteins

MM: Mineralisation Medium

ON: Osteonectin

OP: Osteopontin

Pa: Pascal

PBS: Phosphate Buffered Saline

Pen/Strep: 100U/ml Penicillin/ 0.1mg/ml streptomycin solution

rhVEGF: Human VEGF165

RNA: ribose nucleic acid

RT-PCR: Reverse Transcriptase-Polymerase Chain Reaction

SD: Standard deviation

SPTA: Spatial-peak temporal-average

TGF: Transforming growth factor

TGF-β: Transforming Growth Factor Beta

TF: Transcriptional Factor

US: Ultrasound

VEGF: Vascular endothelial cell growth factor A

W: Watt

W/cm^2: Watt per centimetre squared

CHAPTER 1 INTRODUCTION

1.1 MEDICAL APPLICATIONS OF LOW FREQUENCY ULTRASOUND

The use of ultrasound (US) for biomedical applications is expanding. While US technology is well described in a clinical setting as a diagnostic tool (Denier 1946; Wild 1950; Howry and Bliss 1952), it is the therapeutic benefits of US, such as facilitated drug delivery and healing of soft tissues and bone, which is creating interest for use in tissue engineering and regenerative medicine.

Indeed the application of US therapy dates back to the 1920's when it was first recognised that a 1-2 minute exposure of US could rupture Spirogyra and induce the death of small fishes and frogs (Wood and Loomis 1927). The biophysical effect of US have been well documented (Barnett *et al.* 1994; Gallo *et al.* 2004) and the potential use of US in therapy resulted in many significant studies within a range of biomedical fields including osteology, oncology, pharmacology and molecular biology. These studies have increased our understanding in the biological, chemical and physical effects of US on biological tissues and have identified US conditions for therapeutic and research application. Notably, clinical therapeutic conditions have been developed in a range of clinical areas including physiotherapy (ter Haar 1999), transdermal drug delivery (Mitragotri *et al.* 1996), thrombolysis (Everbach and Francis 2000) and cancer treatment (ter Haar *et al.* 1991) (**Table 1**).

Table 1 US conditions used for a range of therapeutic areas (Shaw and Hodnett 2008)

Therapeutic Area	Treatment Regime (Frequency and intensity of US applied)
Physiotherapy	1-3 Megahertz (MHz); 1 Watt per square centimetre (W/cm$^{2)}$
Lithotripsy	0.5MHz; very low, >20 Megapascals (MPa)
Soft Tissue Lithotripsy	0.25MHz; very low, 5-30MPa
High Intensity Focused Ultrasound (HIFU)	0.5-5MHz; 1000-10,000W/cm^2, 10MPa
Haemostasis	1-10MHz; 100-5000W/cm^2, 5MPa
Bone Growth Stimulation	1.5MHz; 30mW/cm^2, 50kPa
Drug Delivery	Up to 2MHz; various, 0.2-8MPa

The majority of clinical US therapies that have been developed are based on the interaction between the physical effects of US and the cells and tissues, such as controlled disruption of various biological barriers including cell membranes and tissues for drug and gene delivery (Mitragotri 2005; Paliwal and Mitragotri 2006). In contrast, relatively little is known about the more subtle biological effects of US on cells and tissues (Paliwal and Mitragotri 2008). Few studies have been published on how US parameters such as frequency, intensity and treatment time may impact cell function therefore only limited US conditions have been described. Further research

in this field will identified optimised US parameters for maximal stimulation in specific cells and tissues.

1.2 BIOPHYSICAL CHARACTERISTICS OF LOW FREQUENCY ULTRASOUND

US can be defined as sound waves possessing a frequency that is above the limit of human hearing range, usually between 16 to 20 kHz (Williams 1983b). The unit of US is the Hertz (H), which is equal to one cycle per second. Being a propagating pressure wave, US is capable of transferring mechanical energy into tissues, which is then absorbed, propagated or reflected depending on the frequency (ter Haar 1978).

Data indicates that the biological effects of US are primarily dictated by three parameters; frequency, intensity and treatment time (Duarte 1983; Harle *et al.* 2001a). These three factors can be altered to affect the dose thereby changing the total amount of energy emitted from the US beam (Williams 1983b). Other US parameters may also alter the characteristics of the US wave thereby affecting the amount of energy that reaches a specific site (Tsai *et al.* 1992; Reher *et al.* 2002)(**Table 2**). These variables must be considered and tightly controlled when designing and executing US experiments *in vitro* and *in vivo*.

Table 2 Parameters affecting US dose delivered to target tissue

Factors affecting the Energy Profile of the Ultrasound Beam
Ultrasound frequency
Wavelength
Intensity
Amplitude
Effective radiating area of transducer head
Beam non-uniformity ratio (BNR)
Continuous/pulsed therapy
Coupling medium
Tissue composition
Movement and and angle of transducer
Frequency and duration of treatment sessions

US frequency determines the depth at which the US wave can travel and the size of the effected area. Altering US intensity will change the energy of the US beam and therefore the strength of the acoustic wave. Temperature rise in exposed tissues is also dependent on intensity; the higher the intensity, the more exaggerated the temperature rise within the target and surrounding tissues. **Table 3** provides terminology and definitions relating to the characteristic properties of US.

Table 3 Terminology used in therapeutic US (Nyborg 1987)

Term	Definition	Units (If Applicable)
Acoustic Frequency	Number of cycles per second	Hertz (Hz): one cycle per second Kilohertz (kHz) = 1000Hz Mega Hertz (MHz) = 1000kHz
Acoustic Pressure	Deviation (in a sound field) of the pressure from its original value	Pascal (Pa) Megapascal (MPa) = 1,000,000Pa
Temporal Peak Pressure	Greatest value of acoustic pressure during a cycle	N/A
Acoustic Power	Acoustic energy produced per second	Watt (W) Milliwatt (mW) = 0.001W
Acoustic Intensity	Acoustic energy transmitted across unit area per second	Watts per square centimetre (W/cm^2) Watts per square meter (W/m^2) Milliwatts per square centimetre (mW/cm^2) $1W/cm^2 = 10,000W/m^2$ $1mW/cm^2 = 10W/m^2$
Attenuation	Progressive loss of energy during passage through tissue	N/A
Spatial-peak	Intensity at the focus,	

temporal-average (SPTA) intensity (focused beam)	average over time	N/A
Standing Wave	Created when reflected US meets further waves being transmitted, with potential adverse effects on tissue	N/A

US can be divided into three categories in medicine which are Diagnostic US, Disruptive US and Therapeutic US. Diagnostic US utilises high frequencies (between 3-5MHz) and low intensities (1 to 50mW/cm^2) whereas disruptive US, such as those used in ultrasonic cleaning, uses low frequencies (20 to 60kHz) and high intensities above 8W/cm^2. Historically, therapeutic US has a frequency in the range of 0.75MHz to 3MHz but can be further subdivided into applications that utilises non-destructive higher frequencies (between 1 and 3MHz) at various intensities depending on treatment type (5 to 50mW/cm^2) and low frequencies (around 45kHz) at various intensities.

The frequencies most frequently used within the therapeutic range lie between 1-1.5MHz as the US is able to penetrate deeper into tissue structures. The depth of US penetration is indirectly proportional to frequency thus at higher frequencies, US

affects superficial tissues rather than the underlying tissue as available energy is lost due to US absorption.

Other factors affecting the level of absorption of US energy in tissue include beam divergence and energy reflection. Frequency also determines beam divergence (Ward and Robertson 1996). Within the MHz range, the US beam are cylindrical in shape with a small divergence (**Figure 1-1**) and as the US frequency decreases, the beam divergence increases reaching a maximum divergence at a frequency of approximately 73kHz where beam divergence is at an angle of 90°(Ward and Robertson 1996). At frequencies lower than 73kHz, the beam remains completely divergent, which enables a larger treatment area however more energy is reflected from the treatment area rendering the US beam less effective (Ward and Robertson 1996).

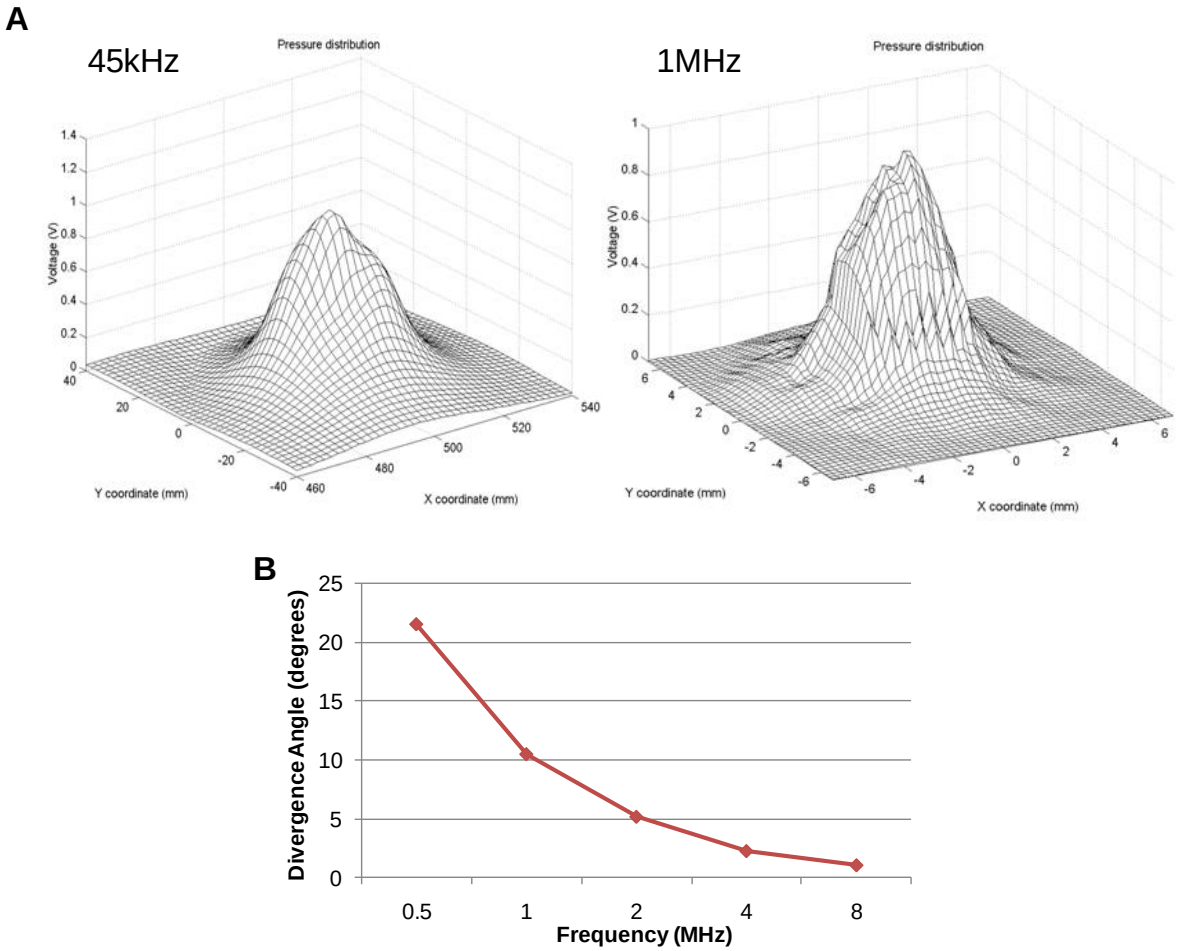


Figure 1-1 US beam shape characteristics of a (**A**) 45kHz and 1MHz transmitter (DuoSon, SRA developments, UK). (**B**) Beam divergence is inversely proportional to US frequency (adapted from Hendee & Ritenour, 2002).

The penetration depth at 45kHz is between 20 and 34 times larger than 1MHz US (Goss *et al.* 1979) enabling deeper tissue penetration. Combined with low intensities, US is able to exert its effects in deeper tissues structures such as bone without inducing temperature increases. Although tissue heating is accepted as a key mode of action for US, evidence in bone fracture healing and soft tissue repair has

highlighted the influence of non-thermal effects, and moreover the mechanical effects of US in stimulating accelerated tissue repair (Webster *et al.* 1978; Dyson 1982; Stewart and Stratmeyer 1982).

1.2.1 THERMAL EFFECTS OF ULTRASOUND

In vivo, US is absorbed by tissues and converted into heat energy (Schortinghuis *et al.* 2003). When US traverses through a tissue, vibrating forces are applied to tissue components such as intra-and extracellular fluids and cell membranes (Williams 1983b). The degree of heat generation is dependent on various parameters including US wave form (continuous or pulsed), and duration of stimulation but is primarily dependent on the intensity of the US beam (Knight and O Draper 2007).

There is a direct relationship between tissue temperature and the protein content of the tissue; the higher the protein content, the more readily US is absorbed and thus the greater the thermal effect (Love and Kremkau 1980; Stewart and Stratmeyer 1982). Notably, muscle tissues yield to the thermal effects of US more readily than fatty tissues and the largest thermal effect occurs in layers adjoining reflecting tissue structures such as bones, joints, cartilage and tendons (Miller and Ziskin 1989; Huang *et al.* 1999; Locke and Nussbaum 2001).

Ideally, to study the direct biological effects of US *in vitro*, temperature needs to be tightly regulated and maintained at physiological temperature of 37°C. Temperature fluctuations increase the chance of perturbations and may produce a host of biological responses that could potentially mask or enhance the cellular effects induced by US exposure. In addition, significant temperature increases may induce cell death. In cell culture studies, temperature increases of 5°C above that of physiological temperature have been extensively cited within the literature to induce heat shock proteins (HSP) (Wagner *et al.* 1996; Amano *et al.* 2006) and the presence of these molecules is regarded as an early indicator of cellular damage (Nussbaum and Locke 2007a).

Continuous wave US administration results in the most significant increases in temperature thereby to reduce US-induced temperature rise of exposed tissues, US can be delivered by pulsed mode, where the acoustic wave is emitted intermittently rather than continuously. In addition, frictional heating can be avoided by using a coupling medium, which also enables acoustic wave transmission to the target area (Warren *et al.* 1976; Casarotto *et al.* 2004). In physiotherapy and US imaging, the contact medium takes the form of an aqueous gel or water (Casarotto *et al.* 2004). For *in vitro* research, cell culture medium is used as the contact medium for US propagation (Loch *et al.* 1971; Reher *et al.* 1997).

1.2.2 NON-THERMAL EFFECTS OF ULTRASOUND

Where thermal effects are negligible, for instance when applying US at low frequencies and low intensities, US is most likely to deliver its biological effects through non-thermal mechanisms (Dyson 1982). Non-thermal US is a combination of acoustic microstreaming and cavitation and applications, such as dental scaling and ultrasonic cleaning, rely upon the destructive nature of these forces to fulfil their clinical role. Non-thermal effects of US however may result in damage to exposed cells and tissues and to minimise these deleterious physiological effects certain US parameters such as intensity may be modified to promote beneficial responses such as tissue regeneration, bone healing and angiogenesis (Young and Dyson 1990).

1.2.2.1 Acoustic Microstreaming

Acoustic microstreaming is the fluid shear forces produced by the oscillatory behaviour of minute bubbles containing gas or vapour caused by the excitation of the acoustic wave (Williams 1983b). This phenomenon occurs when the momentum from the directed propagating sound waves are transferred to the liquid causing the liquid to flow in the direction of the sound propagation (Pitt and Ross 2003). As US intensity increases, the occurrence of acoustic streaming also increases. Acoustic streaming is extremely beneficial to applications such as drug delivery as fluid disruption is sufficient to allow cell membranes to be more permeable to foreign particles. However biologically, irreversible damage to the cell may occur when acoustic streaming is so severe that spontaneous cavitation occurs (Miller and Thomas 1995).

1.2.2.2 Cavitation

Notably acoustic cavitation refers to US generation of air or gas bubbles under excitations of acoustic waves (Wu and Nyborg 2008). Cavitation may result in the formation of micro-bubbles that are unstable and which rapidly collapse thereby releasing large amounts of energy resulting in shearing and microstreaming fields (Watson 2000). The energy produced can continue throughout the exposure period and is sufficient to disrupt chemical bonds and produce chemically reactive free radicals that may cause molecular damage to DNA and proteins (Barnett *et al.* 1997). In contrast, stable cavitation involves the continued pulsation of pre-existing gas-filled bubbles without collapse, and with capabilities for exerting mechanical stress without accompanying chemical action involving free radicals. There is a general consensus within the literature that the biological actions of low frequency US *in vitro* is a consequence of the stimulatory “micro-massaging” of cells by a combination of subtle stable cavitation and microstreaming effects (Webster *et al.* 1980; Sun *et al.* 2001; Tran *et al.* 2007).

1.2.2.2.1 Standing Waves

The creation of a standing waves *in vitro* are the result of acoustic impedance differences between the vessel walls containing the experimental material, and that of the vessel contents (Miller *et al.* 1996). This phenomenon is also produced in the treatment of tissues that are located adjacent to a reflective surface such as at the brain/bone interface (O'Reilly *et al.* 2010). These waves are highly efficient at

concentrating the potentially hazardous effects of ultrasonic energy (Goldman and Lepeschkin 1952).They not only increase the peak pressures delivered, but can create regions of high pressure causing undesired heating (Connor and Hynynen 2004), promote unstable cavitation events (Kerr *et al.* 1989) and present problems in US dosimetry (Kaufman and Miller 1978). To minimise the occurrence of standing waves, using an exposure vessel made of sonolucent material, lowering the US intensity, degassing fluids as well as using acoustic absorbent material to line any reflective surfaces may be applied to the experimental design (Robertson and Ward 1997).

1.3 DENTINE REGENERATION

An important goal in dental research is the efficient regeneration of tooth component tissue. Strategies for natural regeneration of dental tissues as opposed to repairing lost tissue structure will alleviate the current burdens of restorative dentistry including the cost of treatment and replacement of failed restorations due to the longevity of traditional dental materials (Smith 2004). In addition, a significant number of restored teeth will eventually undergo pulpal necrosis requiring either tooth extraction or endodontic treatment (Spinas 2004; Krifka *et al.* 2011). Development of novel techniques to regenerate specific tooth components would therefore have significant societal benefits (Meyer 2009). Cells resident within the tooth have innate regenerative properties and exploitation of these properties may lead to the development of new future clinical therapies in dentistry.

1.3.1 THE DENTINE-PULP COMPLEX

Dentine and pulp are closely related embryologically, developmentally and functionally and thus are frequently referred to as the dentine-pulp complex. Not only do the cells of the pulp core maintain tissue homeostasis after tooth development, but they also underpin the defence reactions taking place in response to injury and the reparative events responsible for tissue regeneration (Smith 2003). The sensitivity of the dentine-pulp complex to injury is one of the key features that make the tooth such a dynamic tissue. It is important to note that the interplay and relative balance among dentine and pulp will be the primary determinant of tissue vitality and tooth survival (Smith 2003).

The dental pulp is a highly vascularised connective tissue (Logan and Nusse 2004) and plays several important functions roles in maintaining tooth vitality. The vasculature provides a supply of nutrients for dentine-pulp metabolism and the pulp is also highly innervated. Combined with its innate immune defence mechanisms the dentine pulp-complex is able to detect and respond to injury by initiating the formation of reparative or reactionary dentine (Nakashima 2005).

The cells of the dental pulp comprise of mainly fibroblasts, defence cells including mainly dendritic cells and histiocytes/macrophages as well as a few lymphocytes and mast cells. Most importantly, the pulp contains undifferentiated mesenchymal cells

(Moss-Salentijn and Hendricks-Klyvert 1990). These stem cells enable the pulp to repair both the hard and soft tissue, which may become compromised due to infection, trauma and chemical insults (Moss-Salentijn and Hendricks-Klyvert 1990).

1.3.2 ODONTOBLASTS AND DENTINOGENESIS

Odontoblasts originate from neural-derived cells of the papilla and are the cells responsible for producing dentine. These cells are terminally differentiated and line the entire pulpal aspect interfacing with the tissue they have formed (Linde and Goldberg 1993). If odontoblasts are lost due to dental disease or trauma, undifferentiated cells must be recruited from the pulp to replace them to repair the damaged dentine. The odontoblast cell body is located at the periphery of the pulp whilst its cellular process extends into the dentine and is enclosed within the dentinal tubules. The odontoblast process enables the secretion of matrix components during mineralisation (Berkovitz *et al.* 2005) and also allows communication between the pulp and the dentine (**Figure 1-2**).

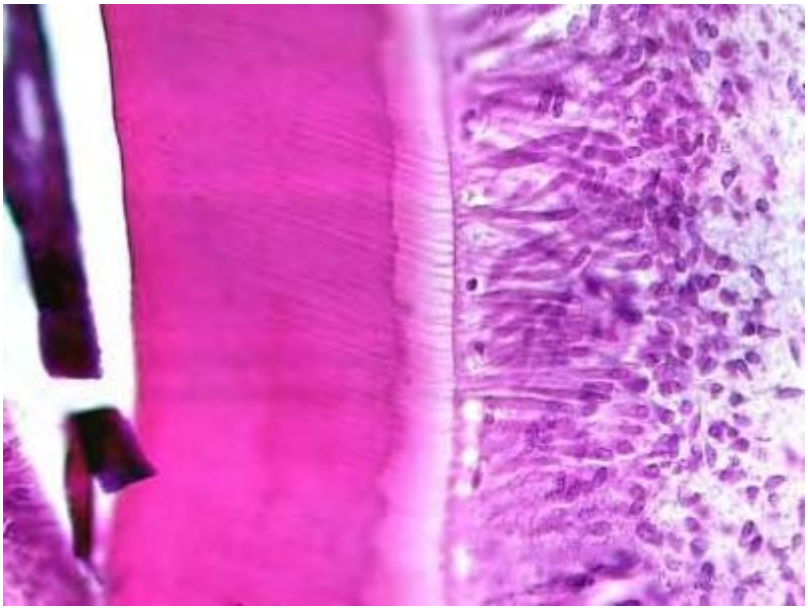


Figure 1-2 H and E stained section of the pulpodentinal region. The pre-dentine lies in-between the odontoblast layer (purple) and the dentine (pink). Polarised odontoblasts line the pulp-predentine junction and their processes are seen extending into the pre-dentine and dentine within dentinal tubules (http://flylib.com/books/en/2.953.1.21/1/).

The morphology of odontoblasts reflects their functional activity. Pre-odontoblasts are small ovoid cells that exhibit poorly-developed organelles and have no specific orientation, which reflects their function that is to solely develop the intracellular machinery needed for active protein synthesis and secretion (Couve 1986). Secretory odontoblasts are elongated; columnar cells and its shape reflect the crowding of the cells as they move in a pulpal direction during dentinogenesis. They are highly polarised and contain numerous organelles necessary for its highly

secretory role (Nanci 2003). Generally, the cells exhibit an oval nucleus with one or two nucleoli and numerous organelles reside in the cytoplasm including rough endoplasmic reticulum, a well-developed golgi apparatus, mitochondria, vesicles, vacuoles, electron dense bodies, microtubules and fibrils. The appearance and frequency of these organelles depend on the functional activity of the cell. The odontoblast process has fewer cytological features than the cell body and is reflective of its secretory role therefore is devoid of any synthetic organelles within the cell body.

The formation of dentine is referred to as dentinogenesis of which 3 physiologically different types are laid down. Primary dentine is rapidly laid down during tooth development and the process of primary dentinogenesis can be divided into three stages: I) cytodifferentiation, in which pre-odontoblasts differentiate into post-mitotic odontoblasts by growth factor signalling particularly those belonging to the Transforming Growth Factor-beta (TGF-β) family . II) Dentine matrix formation whereby the newly formed odontoblasts are actively involved in the synthesis, secretion and reabsorption of collagen and non-collagenous matrix components including dentine sialophosphoprotein (DSPP), dentine sialoprotein (DSP) and dentine matrix proteins (DMPs) to form unmineralised matrix and III) mineralisation, the accumulation of calcium and phosphate to form hydroxyapatite, which occurs within membrane-bound matrix vesicles located in the unmineralised matrix (Bernard 1972). Initial hydroxyapatite crystal formation, often termed nucleation, is a complex

process and thought to be initiated by the attraction of calcium and phosphate ions to dentine phosphoproteins (DPPs), which are bound to a structured three-dimensional (3-D) meshwork of collagen fibres (Goldberg *et al.* 1995). The production of dentine by the odontoblasts occurs as these cells retreat from the dentino-enamel junction towards the centre of the tooth (Moss-Salentijn and Hendricks-Klyvert 1990).

Primary dentinogenesis continues until the teeth are fully developed and thereafter dentine formation proceeds as secondary dentinogenesis throughout the biological life of the tooth (Linde and Goldberg 1993). The deposition of secondary dentine occurs at a slower rate than primary dentinogenesis which results in a gradual reduction in the size of the pulp. At this stage, the odontoblasts exhibit less developed synthesis organelles which reflect their reduced secretory role (Arana-Chavez and Massa 2004). Lastly, tertiary dentine formation is laid down after injury to the tooth and is described in more detail within the following sections.

1.3.2.1 Dentine Composition

Dentine comprises of an inorganic element of mainly hydroxyapatite, and an organic matrix mainly constituted of type I collagen and non-collagenous proteins (NCPs) such as proteoglycans, glycosaminoglycans and lipids (ground substance) and growth factors. The NCPs present within dentine comprise only a minor percentage of the organic matrix compared to collagen however the roles of these proteins is of significant importance. Indeed, these molecules are reportedly sequestered in the

dentine matrix from where they may be released to stimulate repair processes (Roberts-Clark and Smith 2000a; Logan and Nusse 2004; Clevers 2006).

NCPs are synthesised by fully differentiated odontoblasts that occupy the space between the collagen fibrils and the periphery of the dentinal tubules. Acidic NCPs like osteopontin (OP), bone sialoprotein (BSP), osteonectin (ON), osteocalcin (OC) and dentine matrix protein 1 (DMP1) have been implicated in playing an important role in matrix mineralisation through regulation of crystal size (Linde and Goldberg 1993). Additional proteins such as DPP and DSPP that are generally regarded as the main hallmarks of odontoblasts (Ritchie and Wang 1996; MacDougall *et al.* 1997; Ritchie and Wang 1997) are also important in controlling the initiation of matrix mineralisation and the rate of mineral deposition (Berkovitz *et al.* 2005).

Dentine represents a significant storage site for a range of growth factors including insulin-like growth factor (IGF)-I, IGF-II, platelet-derived growth factor (PDGF), epidermal growth factor (EGF), vascular endothelial growth factor (VEGF), placenta growth factor (PlGF), fibroblast growth factor (FGF), metalloproteinase (MMP) and transforming growth factor (TGF) superfamily members such as TGF-β. The presence of these growth factors emphasise the biologically active nature of the matrix and there is evidence that implies the interactions between these molecules may contribute to the induction of pulp and dentine repair mechanisms (Rutherford *et al.* 1993; Nakashima 1994; Sloan and Smith 1999). It is envisaged that the manipulation of these bioactive molecules to promote dental hard tissue production

will ultimately provide new clinical approaches to biologically directed dental tissue repair.

1.3.2.2 Response of the Dentine-pulp complex to Injury

Tertiary dentine is produced in response to various external stimuli such as dental caries, attrition and trauma and takes forms as two subtypes: reactionary and reparative tertiary dentine (Nanci 2003) (**Figure 1-3**). Both however serve the same purpose, which is to protect pulp vitality by producing a barrier between site of injury and the pulpal cells.

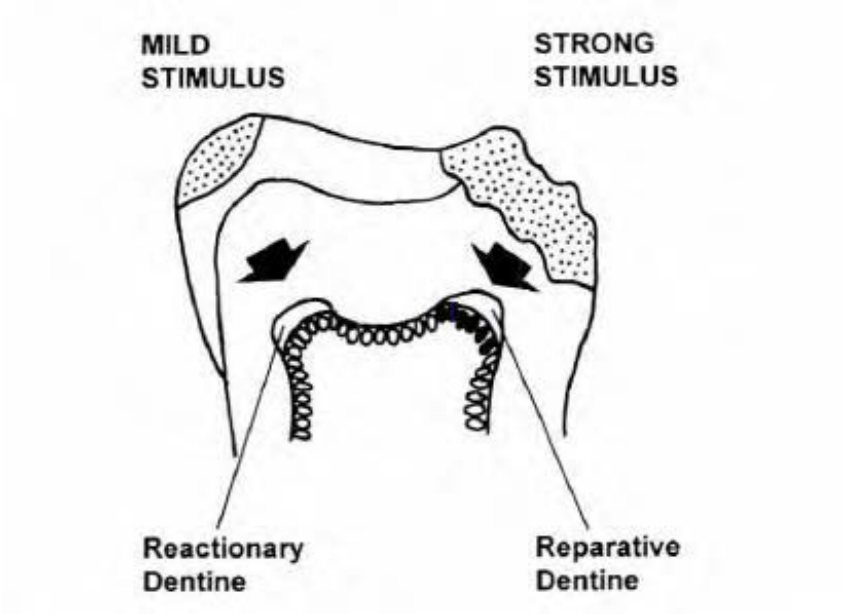


Figure 1-3 Schematic illustration of dentine deposition during tertiary dentinogenesis after mild (i.e. wear) and strong (i.e. trauma) stimuli (Smith *et al.* 1995a).

Reactionary dentinogenesis refers to dentine formed by the existing odontoblasts in response to a mild or moderate insult such as slowly progressing carious lesions or tooth wear (Berkovitz *et al.* 2005). A key feature of this response is that no cell replenishment is necessary or occurs as the odontoblasts survive the injury (Goldberg and Smith 2004). In contrast, reparative dentine relates to dentine produced after severe injury such as rapidly spreading caries or in the case of trauma, a broken tooth (Linde and Goldberg 1993). In reparative dentinogenesis, the original odontoblasts underlying the damaged lesion are destroyed and new mineralised tissue is formed following the recruitment of progenitor cells from within the pulp to form newly differentiated cells or odontoblast-like cells at the site of injury (Berkovitz *et al.* 2005).

The exploitation of tertiary dentinogenic events in clinical practice within restorative dentistry already occurs in pulp capping with calcium hydroxide-based materials (Gronthos *et al.* 2000). Further studies using animal models (Ohshima 1990) and *in vitro* systems (Tsukamoto *et al.* 1992; Hanks *et al.* 1998b; About *et al.* 2000) have now highlighted a range of bio-active molecules present within the dentine that act as promoters of biomineralisation that can be released by certain pulp capping agents. Subsequently, it has been proposed that these bioactive molecules stimulate natural dental tissue repair events (Sloan and Smith 1999; Dobie *et al.* 2002).

1.3.3 HARD AND SOFT DENTAL TISSUE REGENERATION BY TISSUE ENGINEERING

Restoration of dental tissues, lost as a result of trauma and disease, using natural biological materials have significant advantages over current restorations being that the tissue would functionally integrate with the healthy tissue thereby maintaining its health and integrity (Onyekwelu *et al.* 2007). Current dental restorations for the management of carious loss of dentine utilises a range of materials including amalgams, cements and resin composites to provide a physical barrier to maintain tooth function and integrity however, the lifespan of these materials are limited and strategies to development therapies that will promote natural tissue repair are in progress (Smith *et al.* 2008; Cooper *et al.* 2010).

The benefits of exploiting the natural regenerative capabilities of the dentine-pulp complex have already been established (**Section 1.3.2.2)**. The bioengineering of dentine regeneration to promote the deposition of a protective hard-tissue barrier between the pulpal cells and the injury is only one of several approaches for the repair of dental hard tissues and eventually whole tooth regeneration (Du and Moradian-Oldak 2006). Bone repair and regeneration may be stimulated to accelerate and maximise bone ingrowth to treat periodontal and endodontic defects including nonsurgical debridement of root surface or root canals as well as to improve implant osseointegration (Bashutski and Wang 2009). Bone and dentine are both mineralised tissues that closely resemble each other in composition and

mechanism of formation (Qin *et al.* 2004). To help promote bone and dentine repair and regeneration, the local application of growth factors/cytokines and host modulating agents are being used to maximise the healing potentials of these tissues.

1.3.3.1 Signalling Molecules for Hard Tissue Repair

Considerable research has focused on determining the key roles played by growth factors in physiological odontoblast differentiation and the recapitulation of these events during dental tissue repair. Bone and dentine are both mineralised tissues that closely resemble each other in composition and mechanism of formation (Qin *et al.* 2004). Both share a common bank of NCPs that actively promote mineralisation enabling odontogenesis and osteogenesis.

The release of growth factors from the dentine matrix is regarded as key to its bio-activity (Smith *et al.* 1995b). Studies have analysed the role of TGF-β and BMP family members, as well as a number of angiogenic growth factors for their ability to mediate cellular responses to injury in the dentine-pulp complex (Finkelman *et al.* 1990; Lianjia *et al.* 1993; Roberts-Clark and Smith 2000b). Some of these have also been shown to effect the development and behaviour of odontoblast-like cells maintained in culture (Tziafas *et al.* 1995; Roberts-Clark and Smith 2000a; He *et al.* 2004). Both TGF-β and BMP family members have been shown to be active signalling molecules during both primary and tertiary dentinogenesis (Finkelman *et*

al. 1990; Smith *et al.* 1994). Indeed TGF-β1 has been shown to directly upregulate odontoblast activity and stimulate reactionary dentinogenesis when directly applied to odontoblasts (Sloan and Smith 1999) whilst BMP-2 and BMP-7 are able to stimulate reparative dentine formation (Rutherford *et al.* 1993; Nakashima 1994). Furthermore, BMP-2, BMP-7, TGFβ-2 and insulin-like growth factor have been shown to enhance bone formation for the osseointegration of implants (Lynch *et al.* 1991; Sumner *et al.* 1995; Koempel *et al.* 1998; Lind *et al.* 2000) and for mediating periodontal attachment (Ripamonti and Reddi 1997).

Angiogenic events are also a prerequisite for bone and tooth development and repair (Baume 1980). Both PDGF and VEGF are potent angiogenic mitogens present within the dentine matrix (Roberts-Clark and Smith 2000b) that are subsequently released following tooth injury, caries and/or dental restorations for the regulation of dental pulp repair (Smith *et al.* 1990; Grando Mattuella *et al.* 2007; Zhang *et al.* 2011). VEGF is regarded as the most important growth factor for regulating vascular responses within the body and its expression in pulp cells can occur due to hypoxia, inflammation and in response to orthodontic forces or trauma (Lau *et al.* 2006; Robinson *et al.* 2006; Sawakami *et al.* 2006). VEGF mRNA is also expressed in odontoblasts (Scheven *et al.* 2009a) and dental pulp fibroblasts (Takashi Yamashiro 2007). Studies support the fact that angiogenic responses during tertiary dentinogenesis is regulated by synergistic effects from a combination of growth factors and signalling molecules (Derringer and Linden 2004). VEGF also plays a significant role in angiogenesis for osteogenesis. When encapsulated in hydrogel

microspheres, VEGF was able to stimulate enhanced vascularisation in chondrocytes (Murphy *et al.* 2000; Peters *et al.* 2002) and in combination with BMP-2, enhanced bone regeneration was observed on the repair of bone defects around dental implants (Luo *et al.* 2011).

1.4 ENHANCEMENT OF TISSUE REPAIR BY LOW FREQUENCY ULTRASOUND

US at a broad range of frequencies and intensities can influence biochemical pathways by inducing signals to enhance cell function such as cell proliferation rate (Reher *et al.* 1997; Doan *et al.* 1999). For cells to regenerate they must be stimulated to proliferate and the processes required for tissue replacement must be induced. Studies as early as 1950 initially indicated that US could stimulate osteogenesis (Buchtala 1950) and the use of low intensity pulsed US (LIPUS) to accelerate fracture healing is now well established (Han *et al.* 2001; Cooper 2002; Han *et al.* 2002). Indeed it has been previously demonstrated that LIPUS stimulates bone and cartilage cells to increase the production of growth factors and tissue matrix thereby promoting hard tissue healing (Reher *et al.* 1998; Zhang *et al.* 2003; Claes and Willie 2007). Notably in experiments using rat fibulae, healing was shown to be accelerated when LIPUS treatments were performed during inflammatory and early proliferative phases of bone repair following fracture (Nussbaum and Locke 2007a).

LIPUS has also been suggested as a non-invasive and locally applied strategy for enhancement of endogenous bone regeneration around a dental implant (Tanzer *et al.* 2001; Dimitriou and Babis 2007). Furthermore, LIPUS may also be a useful tool for Class II malocclusion and mandibular deficiencies where it has been reported to induce bone growth modifications of the mandibular condyle (El-Bialy *et al.* 2006). More recently, US has been shown to have a positive effect on stem cells (Lee *et al.* 2006; Lee *et al.* 2007) and may potentially be advantageous for stem cell research and tissue engineering applications.

Biological responses to US are determined by the frequency and intensity of the US applied and different cells show different sensitivities and responses to US due to their intrinsic properties. Interestingly, a well known marker for cell responsiveness to mechanical stimuli is cell morphology (McCormick *et al.* 2006) and both osteoblasts and dental tissue-derived cells have been shown to alter their shape and size when subjected to mechanical strain and stress (Dhopatkar *et al.* 2005; McCormick *et al.* 2006). Moreover, by distorting cell shape, cellular biochemistry and gene expression may also be affected. Indeed in bone cells, pulsed US has been shown to elongate cell shape and alter the alignment of actin fibres (Meazzini *et al.* 1998; McCormick *et al.* 2006).

Changes in the electrophysiological properties and ionic permeability of cell membranes (Dinno *et al.* 1989) have previously been demonstrated in cells exposed

to low frequency US (Parvizi *et al.* 1999). These events may affect synthesis, secretion or motility within the cell, all of which could promote regeneration and healing responses (Dyson and Suckling 1978). Thus far, no studies have been conducted regarding the effect of US on the morphology of dental tissue-derived cells however, when teeth are subjected to mechanical forces during orthodontic tooth movement, similar stresses as those seen in US exposure are transmitted to the dental pulp (Derringer and Linden 2004; Dhopatkar *et al.* 2005) that can then result in the activation of odontoblasts for accelerated dentine mineralisation (Kong *et al.* 2010).

1.4.1 CURRENT EVIDENCE FOR DENTAL TISSUE REPAIR BY EXPOSURE TO LOW FREQUENCY ULTRASOUND

The use of US as a therapeutic tool in dentistry is extensively used in temperomandibular disorder therapy (Erickson 1964) and has generated recent interest particularly within orthodontically-induced tooth-root resorption (El-Bialy *et al.* 2004; El-Bialy 2007; Dalla-Bona *et al.* 2008). Furthermore, US exposure to periodontal ligament fibroblasts, mandibular osteoblasts and gingival epithelial cells has been demonstrated to accelerate periodontal wound healing (Ikai *et al.* 2008). Currently, there is limited data indicating the use of US to enhance dentine-pulp regeneration however, key molecules necessary for tertiary dentinogenesis have been shown to be upregulated in odontoblast-like cells in culture following exposure to low frequency US (Scheven *et al.* 2007). Furthermore, low frequency US is able to

upregulate expression of the angiogenic growth factor, VEGF, and secretion in odontoblast-like cells (Scheven *et al.* 2009a).

It is reasonable to speculate based on reports from other related fields that US has the potential to stimulate dental hard tissue repair. Indeed when osteoblasts are exposed to LIPUS, enhanced growth factor production including interleukin-8 (IL-8), basic fibroblast growth factor (FGF), VEGF, TGF-β1, and alkaline phosphatase (ALP) were observed (Reher *et al.* 1999). These growth factors are also released from the dentine matrix upon dentinogenesis during repair (Roberts-Clark and Smith 2000b). Furthermore, TGF-β1 has been shown to be vital for LIPUS transduction in chondrocytes (Mukai *et al.* 2005) and within nucleus pulpous cells (Kobayashi *et al.* 2009).

1.4.1.1 Dental Ultrasonic Scalers as a Tool for Low Frequency Ultrasound Transmission to Cells *In Vitro*

Currently low frequency US in dentistry is applied at a frequency of ~30kHz and at high intensities for the scaling and root planing of teeth for the treatment of periodontal disease (Webster *et al.* 1980). However, using a dental scaler for low frequency US delivery into biological systems is a novel method for *in vitro* US exposure. Preliminary studies into the application of low frequency US to dental tissues have been reported demonstrating the effects of dental ultrasonic

instrumentation on the viability of primary osteoblasts *in vitro* (Sura *et al.* 2001). In addition, Scheven *et al.* (2007) previously developed dental scaler ultrasonic application of odontoblast-like cells by measuring parameters including US power, US intensities and temperature output generated by the device. It is conceivable that in the future a novel hand-held dental US device may be designed for therapy as well as an added functionality using a multi-frequency transducer to image, diagnose and monitor oral and dental diseases and tissue healing (Scheven *et al.* 2009b).

1.5 INTRACELLULAR SIGNAL TRANSDUCTION: POSSIBLE MECHANISMS OF ACTION FOR LOW FREQUENCY ULTRASOUND

While LIPUS is increasingly used as a supplementary therapy to promote bone and wound healing, the underlying mechanism of action is poorly understood. Experiments involving US at low frequencies and low intensities suggests that the micromechanical strains produced by the acoustic waves is responsible for the biochemical events at the cellular level and that these responses are not due to the thermal side-effects of US (Baker *et al.* 2001). However, the exact mechanism underlying the therapeutic action of US is still unknown. Several theories have been proposed within the literature suggesting as to how the US signal may be converted to biological responses within the cell and proposed mechanisms include i) changes in cell membrane permeability thereby affecting secondary messenger adenylate cyclase activity (Rubin *et al.* 2001), ii) activation of the “stretch receptor” type of cation channel at the cell surface initiating changes in cation concentration thereby

modifying intracellular signals regulating gene expression (Sachs 1991) and iii) increased potentials as a function of US intensity frequency (Behari and Singh 1981; Duarte 1983).

Evidence is however accumulating that indicates transmitted acoustic energy is converted to intracellular biochemical signals via integrin-associated pathways. Integrins are the main membrane receptors that relay extracellular mechanical signals into the cell, which then may activate changes within the cytoskeleton to the cellular matrix thereby affecting cell metabolism and gene expression (Grossi *et al.* 2007). Once the receptors are activated, recruitment of a range of proteins occurs followed by activation of signalling pathways such as the mitogen-activated protein kinase (MAPK) and the integrin/phosphatidylinositol 3 kinase (PI3K)/Akt pathways. Activation of these pathways by LIPUS resulted in enhanced cell proliferation and further stimulated the production of growth factors, which subsequently act by both autocrine and paracrine mechanisms to promote tissue healing (Hauser *et al.* 2008b; Lu *et al.* 2008b; Takeuchi *et al.* 2008; Shiraishi *et al.* 2011). The Wnt/β-catenin signalling pathway is also implicated in both bone tooth development as well as in adult tissue homeostasis (Raz *et al.* 2005) and has been suggested as a key intracellular pathway for US enhanced bone regeneration (Olkku *et al.* 2010). Furthermore, this pathway may also be important for intracellular signal transduction in US-exposed tooth cells.

Wnts form a large family of secreted ligands that activate several receptor-mediated pathways (Hauser *et al.* 2008a). The Wnt signalling cascade initiates at the cell membrane when a Wnt family protein interacts with its receptor, the Frizzled (FZ) receptor and LDL receptor related protein (LRP) family co-receptors. Currently, four different pathways (Fulton 1984) are believed to be activated upon Wnt receptor activation. Of these four, the canonical or the β-catenin pathway is the best characterised. Subsequently the β-catenin pathway is activated and the cytoplasmic dishevelled (dvl) protein is phosphorylated which inhibits glycogen synthase kinase (GSK)-3β. This molecular process results in the accumulation of non-phosphorylated β-catenin in the cytoplasm (Papakonstanti and Stournaras 2008). The unphosphorylated β-catenin then translocates to the nucleus where it releases the inhibitory factor Groucho that activates the transcription of target genes such as COX-2, which has been shown to be rapidly upregulated in the osteoblast cell line, MC3T3-E1 when exposed to low frequency US (Sena *et al.* 2005). Notably, several Wnt genes are broadly expressed in the oral and dental epithelium, while others are upregulated in developing teeth (Bouchet-marquis *et al.* 2007). In early tooth development, several Wnt gene family members are expressed from the initiation stage to the early bell stage (Ivaska *et al.* 2007). Targeted inactivation of lymphoid enhancer factor-1 (LEF1), a nuclear mediator of Wnt signalling, results in the arrest of tooth development at the bud stage (Toivola *et al.* 2005), loss of expression of a direct LEF1/β-catenin target gene, Fgf4 (Pallari and Eriksson 2006), and failure of survival of dental epithelial cells (Pollard 2003).

Other data indicating the importance of Wnt signalling in dental tissue repair derives from data demonstrating that Wnt10a is specifically expressed in the odontoblast cell lineage in mouse molars and co-localises with DSPP mRNA expression that regulates dentine mineralisation in the fully differentiated secretory odontoblast (Cooper 1987). The exact mechanism by which this signalling pathway interplays with other cellular processes however still remains unclear. A better understanding on how various US parameters alter these and other intracellular signalling pathways and influence associated cellular functions will enable better treatment plans to be developed for existing and future US therapies.

1.6 PROJECT AIMS

The aim of this project was to assess *in vitro* osteoblast and odontoblast function in response to low frequency US as a potential tool for dentine-pulp repair using established model cell lines. To achieve this aim, it was necessary;

- To develop a well-defined low frequency US delivery system for the treatment of bone and tooth cells *in vitro*.
- To assess a range of US frequencies, intensities and treatment times to elucidate optimum parameters for *in vitro* low frequency US delivery.
- To evaluate possible mechanisms of action for intracellular low frequency US signal transduction.

The outcome of this project enabled:

- Development of a highly-controlled, calibrated system for applying low frequency US to osteoblast and odontoblast cell models.
- Determination of optimum parameters for low frequency US to induce maximal stimulation of processes pertinent for dentine-repair.
- Enhancement of knowledge for the cellular and molecular mechanisms involved in low frequency US transduction in odontoblast-like cells and its relevance to dentine repair.
- Provision of supportive data for observations seen in current studies using low frequency US as a tool for tissue repair.

CHAPTER 2 GENERAL METHODS

2.1 IN VITRO ULTRASOUND DELIVERY

2.1.1 DENTAL ULTRASONIC SCALER

A MiniMaster piezoelectric generator (EMS, Nyon, Switzerland) was used to produce low frequency US at 30kHz for delivery to cell lines (see **Section 2.2.1**) using an analytic D-tip scaler probe with an attached scaler tip. The scaler tip was immersed into a cell suspension in a 5ml bijou (Appleton Woods, UK) (refer to **Section 2.2.2.1**) and held in position using a clamp and stand ensuring the tip was positioned in the middle of the bijou so it did not contact the walls of the bijou to avoid frictional heating (refer to **Figure 2-1**).

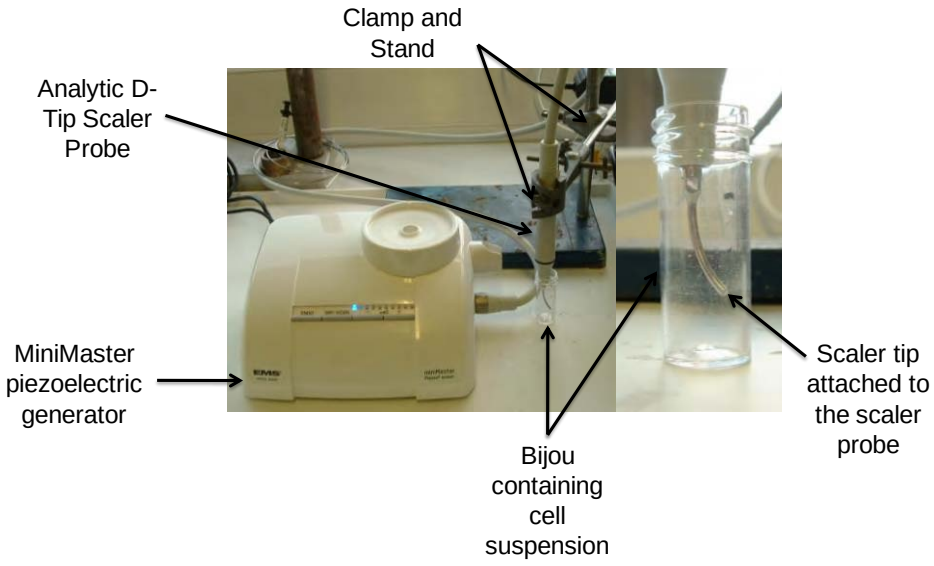


Figure 2-1 Experimental arrangement for US exposure of cells *in vitro* using a dental ultrasonic scaler. (**A**) 30kHz US was generated by using a MiniMaster piezoelectric generator and delivered to cell suspensions using an analytic D-tip scaler probe attached to a scaler tip. (**B**) The scaler tip did not come into contact with the container wall.

Three piezo scaler tip designs of different shapes and sizes were used for US delivery (**Figure 2-2**). Ultrasound was applied at either low, medium or high power settings denoted hereafter as P1, P5 or P7. To assess whether such treatments influenced temperature, 5mls of Dulbecco's Modified Eagle Media (DMEM, Biosera, UK) without glutamine, with 4.5g/l glucose and sodium pyruvate,10% foetal calf serum (FCS, Biosera, UK), 100 Units (U)/ml penicillin/0.1mg/ml streptomycin (pen/strep, Sigma, UK), and 2mM GlutaMAX™-I (Invitrogen, UK) (supplemented DMEM) was exposed to US at P1, P5 and P7. Temperature rise was recorded at 1 minute intervals using a fine wire thermocouple (Iso-tech IDM 207, Isothermal Technology, UK).

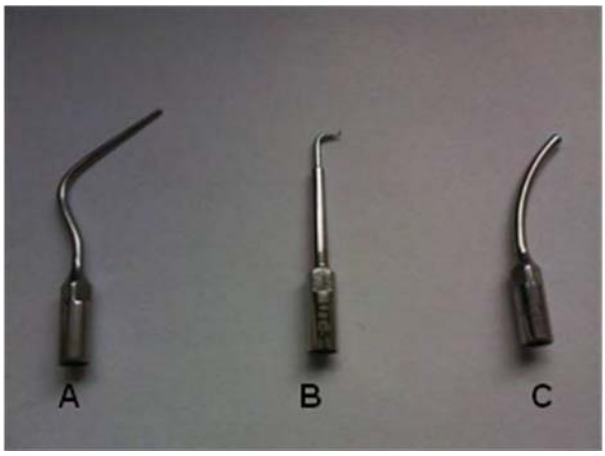


Figure 2-2 Three piezo scaler tips, (**A**) the Angled, (**B**) 078 and (**C**) the D-Tip were used for US delivery to assess their effects on thermal output and cell survival.

2.1.2 DUOSON THERAPEUTIC ULTRASOUND DEVICE

The DuoSon ultrasonic therapy unit (DuoSon, SRA Developments, UK) was calibrated using a radiation force balance to deliver user-defined modes for low frequency US delivery to adherent cell lines *in vitro*. The device was installed with custom intensity settings (**Table 4**) delivering US at 45kHz, 1MHz and at a combined frequency of 45kHz and 1MHz. Calibration was performed annually using a radiation force balance at SRA developments, Devon, UK.

Table 4 Available user-defined settings for the DuoSon Therapeutic Ultrasound Device

Mode	LF Power	LF Intensity	HF Power	HF Intensity	Total Power
1	-	-	20mW	250mW/cm²	20mW
2	-	-	60mW	750mW/cm²	60mW
3	150mW	10mW/cm²	-	-	150mW
4	400mW	25mW/cm²	-	-	400mW
5	400mW	25mW/cm²	20mW	250mW/cm²	420mW
6	1220mW	75mW/cm²	-	-	1220mW
7	1220mW	75mW/cm²	20mW	250mW/cm²	1240mW
8	1220mW	75mW/cm²	60mW	750mW/cm²	1280mW

A novel anti-reflection chamber was designed to house cultures of adherent cells for DuoSon US delivery (**Figure 2-3**) (refer to **Section 2.2.2.2**). The chamber was lined with acoustic-absorbent silicone and moulded to fit a multi-well plate within a

waterbath at the required temperature. As previously reported the silicone lining used would not only absorb reflective acoustic waves thereby minimising the occurrence of standing waves (Hisaka *et al.* 2001), but also served as a heat insulator thereby maintaining the experimental conditions at relatively constant levels and potentially improving reproducibility between experiments.

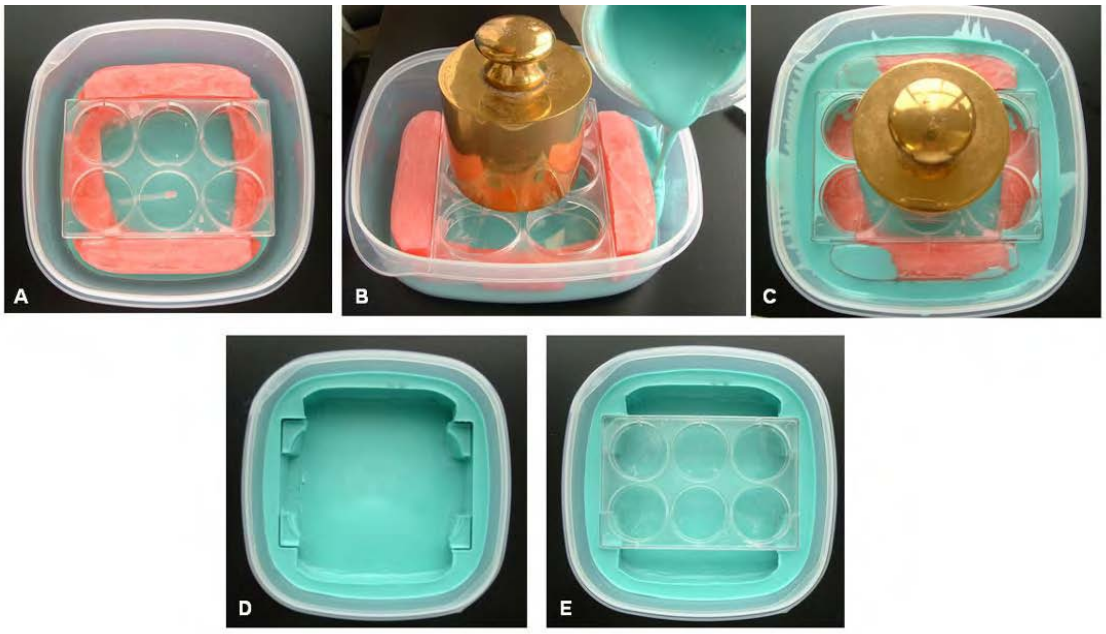


Figure 2-3 Custom-built anti-reflection chamber for *in vitro* US delivery. (**A**) Modelling wax was used to cast a 6 well plate template. (**B**) Liquid silicone impression material was poured into a square box and left to set and (**C**) a 5kg weight was used to enable the desired setting positioning. (**D**) The wax mould was removed to leave the (**E**) end-product design.

Unlike US set-ups used by many other groups, the US in this present study was transmitted to the cells from above, using culture medium as the contact medium (**Figure 2-4**). This approach enabled minimal loss of US intensity to the cells as the acoustic wave would directly transmit to the cells and not through the polystyrene tissue culture plate, which is less acoustically transparent (Doblhoff-Dier *et al.* 1994; Marvel *et al.* 2010) .

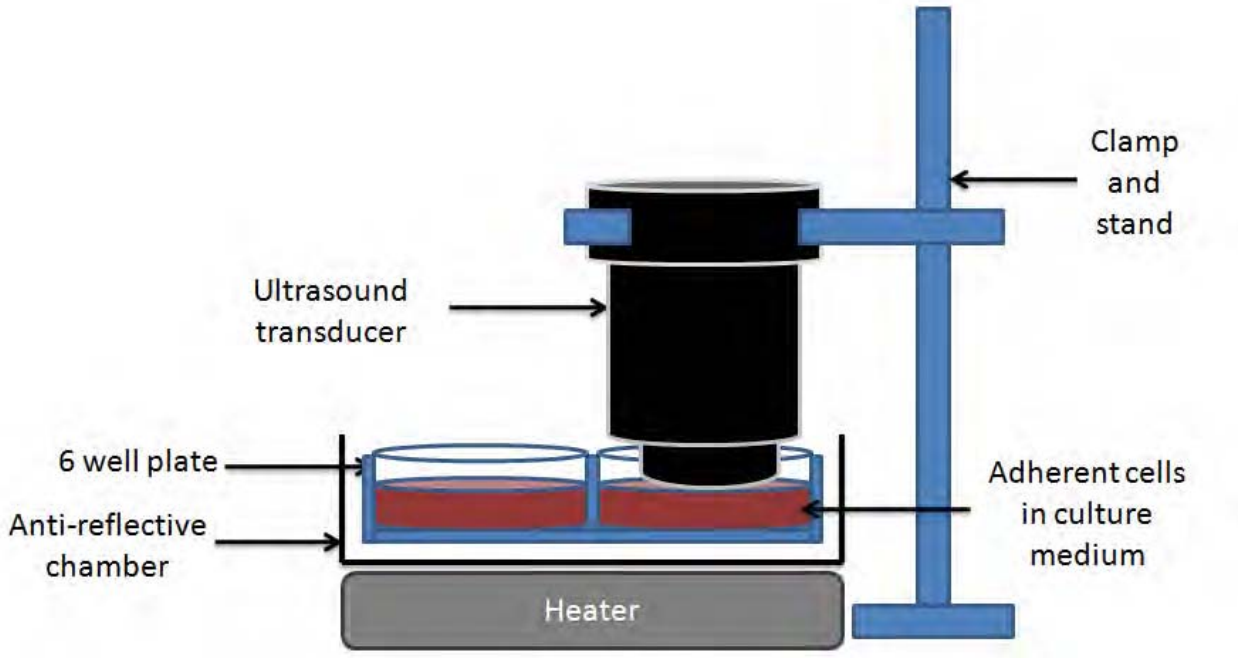


Figure 2-4 Schematic representation of the DuoSon US delivery system. Ultrasound was transmitted to the cells from above.

The anti-reflective chamber was prepared 24 hours prior to use by cleaning the chamber with 70% industrial methylated spirit (IMS) (VWR, UK) and filling with autoclaved deionised water. The chamber was placed into a 37°C incubator (Galaxy

S+, RS Biotech Ltd, UK) with a humidified atmosphere of 95% air, 5% CO2 for 24 hours. Immediately prior to use, the DuoSon, hotplate, clamp and stand were swabbed with 70% IMS and left to air-dry in a laminar flow hood. The temperature of the water within the anti-reflective chamber was maintained at 37°C using a hotplate (Bibby, UK) and the US transducer head was washed with 100% ethanol, allowed to air dry and further rinsed with supplemented DMEM.

Cell lines were seeded onto a 35mm diameter 6-well plate (Appleton Woods, UK) (refer to **Section 2.2.2.2**), which was inserted into the anti-reflective chamber and placed on to the hotplate. The US transducer was then held in place using a clamp and stand and lowered into each well so that it contacted the surface of the medium (see **Figure 2-5**). Ultrasound was delivered to the cells by selecting the required mode and treatment time on the DuoSon device. Experimental temperature was monitored by measuring the temperature of the water bath periodically using a thermometer.

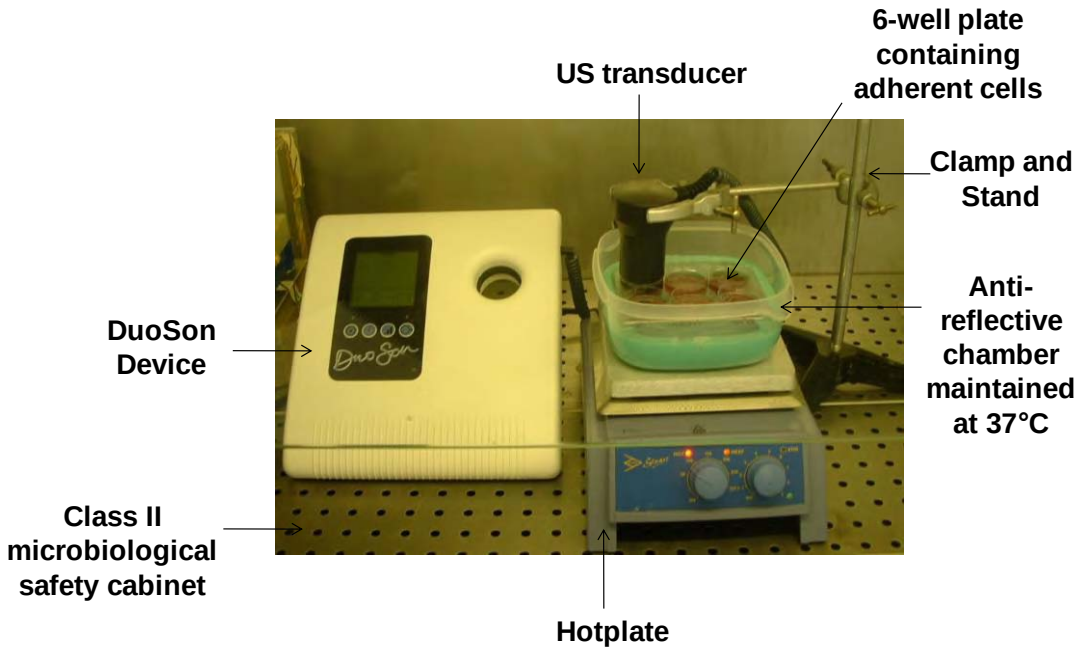


Figure 2-5 Photographic image of assembly of the DuoSon US device adapted for delivery of low frequency US to cells *in vitro*. Adherent cells in 6-well plates were housed in a temperature-controlled anti-reflective chamber for US delivery.

2.2 MAMMALIAN CELL CULTURE

2.2.1 CELL LINES

Cell culture experiments were performed using two murine-derived cell lines: Mouse odontoblast-like cells (MDPC-23) (Hanks *et al.* 1998a) and osteoblastic MC3T3-E1 cells (Sudo *et al.* 1983)(Cat. # 99072810, Health Protection Agency Culture Collections (HPACC), UK). Both cell lines were cultured initially in 25cm^2 tissue culture flasks (Appleton Woods, UK) and subcultured into 75cm^2 tissue culture flasks

(Appleton Woods) for further expansion in supplemented DMEM containing 4.5g/L glucose and sodium pyruvate (Appleton Woods, UK), 10% FBS (Biosera, UK) and 2mM GlutaMAX™-I (Invitrogen). Cell lines were cultured in a 37°C incubator with a humidified atmosphere of 95% air and 5% CO_2.

2.2.2 CELL CULTURE PROCEDURES FOR ULTRASOUND EXPOSURE

2.2.2.1 Dental Ultrasonic Scaler

Confluent cell cultures (**Section 2.2.1)** were detached from plasticware using 2.5g/l of Trypsin in 0.38g/l of EDTA (Invitrogen, UK) and transferred to a 15ml Falcon tube (Appleton Woods, UK) for centrifugation at 1200rpm for 4 minutes to pellet the cells (5804R, Eppendorf, UK). The pellet was re-suspended in supplemented DMEM and pipetted into a 5ml bijou as a homogenous single cell suspension at a density of 100,000cells/ml. Ultrasound at 30 kHz was then applied to the cell suspension for 5 minutes at the three incremental powers previously described in **Section 2.1.1**.

Following US exposure, cell numbers were counted and assessed for viability by transferring 20µl of cell suspension to 20µl of 0.4% trypan blue solution (Sigma-Aldrich, UK) into a 2ml Eppendorf (Appleton Woods, UK). The suspension was gently vortexed (Vortex Genie-2, Scientific Industries, UK) prior to 15µl of the mixture being added to each chamber of a Neubaeur haemocytometer (Neubaeur, Germany). Cells were counted on the haemocytometer visualised using a light microscope (Zeiss

Axiovert 25) and viable (unstained) and non-viable (stained) cells were counted over a primary square of $1mm^2$. Briefly, percentage viable cells were calculated by dividing the number of viable cells by the total number of cells counted and multiplying the total by 100. To calculate cell concentration per ml, the number of cells in a $1mm^2$ primary square was multiplied by 10^4 and the total was then multiplied by the dilution factor.

Alternatively, cell viability and cell proliferation was determined using the WST-1 assay (Roche). Ultrasound-exposed cells were seeded at a density of 5000 cells/well in 100µl of supplemented DMEM onto a flat-bottomed, tissue culture grade 96-well plate (Sarstedt, UK). A standard curve was prepared by serial dilution using the cell lines previously described in **Section 2.2.1** in 100µl of supplemented DMEM. The plate was incubated at 37°C for 12 hours. 10µl of WST-1 reagent was added to each well, briefly agitated and further incubated at 37°C for 30 minutes whilst protecting from light exposure to avoid reagent degradation. Cell viability and proliferation was determined by reading the plate absorbance at 450nm using a spectrophotometer microplate reader (ELx800, Bio-Tek, UK).

2.2.2.1.1 Stimulation of Cell proliferation by Vascular Endothelial Growth Factor

MDPC-23 cells were seeded at a density of 5000 cells/well in 96 well plated in supplemented DMEM and left to adhere in a incubator at 37°C for 24 hours (refer to

Section 2.2.1.). Cell culture medium was aspirated and replaced with supplemented DMEM containing either 1, 10, 50 or 100ng/ml of recombinant human $VEGF_{165}$ (rhVEGF, PrepoTech EC, UK) and cultured for a further 48 hours. rhVEGF conditioned medium was subsequently removed and the cells were washed in DMEM without supplements before cell proliferation was assessed using WST-1 assay as described in **Section 2.2.2.1**. Cell cultures were subsequently returned to the incubator and cell proliferation was assessed by WST-1 assay every 24 hours for a total of 3 days.

2.2.2.2 DuoSon Therapeutic Ultrasound Device

Confluent cell cultures were trypsinised and cells pelleted as previously described in **Section 2.2.1**. The cell pellet was re-suspended in supplemented DMEM and seeded in 6-well plates at a density of 70,000 cells per well. For immunocytochemical analyses, a 22mm diameter coverslip was placed into each well prior to seeding cell lines. Cultures were incubated for 24 hours prior to US exposure at 37°C.

US was applied to cell lines in 6-well plates at a frequency of 45kHz, 1MHz and combined frequency (45kHz and 1MHz) for a single 15 minute exposure or a single 30 minute exposure at each US frequency (refer to **Section 2.1.2**). Daily exposures to US have been reported to be the optimum treatment regime for bone healing and growth stimulation (Tsai *et al.* 1992). To assess cell response to multiple US

exposures, US was applied to the cells 24 hours after the initial US exposure for the same length of time at the same US frequencies. The control wells were handled identically however without US application. Following US exposure, culture plates were returned to the incubator and the cells were collected by the addition of Trypsin-EDTA 48 hours after initial US exposure to assess cell number and viability using Trypan blue staining and counts (see **Section 2.2.2.1**).

2.3 IMMUNOCYTOCHEMISTRY

2.3.1 BETA CATENIN ANTIBODY STAINING FOLLOWING ULTRASOUND EXPOSURE

Cell lines stimulated with US using a dental ultrasonic scaler, as described in **Section 2.1.1** and were seeded at a density of 20,000 cells per well in supplemented DMEM onto 13mm diameter coverslips (Fisher Scientific, UK) placed in the wells of a 24-well plate (Appleton Woods, UK). The plate was then placed in an incubator at 37°C for 4 or 24 hours prior to the cells being washed with sterile 1x phosphate buffered saline (PBS, Sigma, UK) and fixed in 1:3 acetone/methanol (VWR, UK) mixture for 20 minutes prior to beta (β)-catenin staining. Cells exposed to a single 30 minute exposure of US using the DuoSon (**Section 2.2.2.2**) were returned to an incubator at 37°C for 4, 24 or 48 hours. After each timepoint, the cells were washed in 1x PBS and fixed in 1:3 acetone/methanol mixture for 20 minutes prior to staining for β-catenin, as described below (Caldwell *et al.* 2004).

Cells were washed with sterile 1x PBS three times before endogenous peroxidases were blocked by incubating the cells in 0.5% hydrogen peroxide (Sigma-Aldrich, UK) in methanol (VWR) for 30 minutes. The wells were then washed three times with 1x PBS with the last wash lasting 5 minutes. To block non-specific binding, 20% goat serum (Sigma-Aldrich, UK) was added to the cells and incubated for 4 hours at room temperature. Mouse β-catenin primary antibody (BD Bioscience) diluted with 20% goat serum at a ratio of 1:1000 was added to each well and incubated overnight at 4°C. The primary antibody was removed by washing the cells in 1x PBS as described in the previously. Biotinylated goat anti-mouse secondary antibody (Vector Laboratories, UK) diluted 1:200 in 1x PBS was prepared and added to each well and left to incubate for 30 minutes at room temperature.

The avidin/biotinylated, horseradish peroxidase complex was freshly prepared according to manufacturer's instructions using an Elite ABC kit (Vector Laboratories, UK). Following incubation with the secondary antibody, wells were washed in 1x PBS prior to avidin/biotinylated, horseradish peroxidase complex addition to each well and left to incubate at room temperature for 30 minutes. The wells were then further washed with 3 times in 1x PBS as described above.

β-catenin staining was visualised by the addition of chromogenic substrate diaminobenzidine (DAB) (ImmPACT DAB, Vector Laboratories, UK) prepared fresh according to manufacturer's instructions. Each well was then rinsed with 1x PBS until

no trace of brown dye was visible in the wash. Cells were counterstained by rinsing briefly in 1% Mayer's Haematoxylin (Surgipath Europe Ltd) followed by washing in tap water containing 0.1% sodium bicarbonate (Sigma-Aldrich, UK).

The coverslips containing the β-catenin stained cells were then transferred into glass dishes and finally cleared in xylene (Genta Medical, UK) before being mounted onto a microscope slide (Surgipath Europe Ltd) with Xam (a xylene-based mountant) (BDH, UK). Images were captured using the Cool SNAP-Pro *CF* 36-bit colour digital camera (Meyer Instruments Inc., USA) and uploaded to Image Pro Plus Imaging Software (Media Cybernetics, Inc., USA) to correct for background illumination. Subsequently, the images were transferred to ImageJ software (http://rsbweb.nih.gov/ij/) and the frequency of β-catenin positive cells were determined by using the cell counter plug-in (http://rsbweb.nih.gov/ij/plugins/cell-counter.html).

2.3.2 STAINING FOR ACTIN WITH ALEXA FLUOR® 488 PHALLOIDIN FOLLOWING ULTRASOUND EXPOSURE

Visualisation of actin fibres by fluorescence was performed in accordance with manufacturer's instructions in cell lines exposed to a single 30 minutes application of US delivered using the DuoSon (refer to **Section 2.2.2.2**). Briefly, the cells were incubated for 48 hours after US exposure at 37°C and subsequently washed in 1x

PBS and fixed in 10% formalin (VWR, UK). Formalin was then removed by washing the cells twice in 1x PBS before incubating in 0.1% Triton x-100 in 1x PBS for 5 minutes at room temperature. The cells were then washed twice with 1x PBS before the addition of 1% bovine serum albumin (BSA, Sigma-Aldrich, UK) in 1x PBS. The plates were incubated at room temperature for 30 minutes.

After incubation, the cells were washed twice in 1x PBS and incubated with Alexa Fluor™ 488 fluorescent dye conjugated to phalloidin (Invitrogen, UK) diluted to 1:40 with 1% BSA. The wells were protected from light and left at room temperature for 20 minutes. Two further washes with sterile PBS subsequently followed and the stained cells were viewed using an Eclipse TE300 phase contrast light microscope (Nikon). Photomicrographs were captured through a side port with an extension tube using a Coolpix 950 digital camera (Nikon) and subsequently uploaded to ImageJ software for background correction and actin intensity analysis.

2.3.2.1 Image Analysis

To quantify the intensity of actin staining, background intensities were first subtracted from fluorescent photomicrographs using the ImageJ calculator plus plug-in (http://rsbweb.nih.gov/ij/plugins/calculator-plus.html) before the images were converted to a Hue Saturation Brightness (HSB) images, an intuitive colour system that allows measurement of the hue of a colour, the colour's vibrancy and

approximate brightness. Converted images were divided into areas that were black, which represented an absence of fluorescence, and areas of different shades of grey representing areas of fluorescence of different intensities. To assess the overall fluorescent intensity in an image, 100 grey areas per image were manually highlighted and measured for their mean grey value, which is the sum of the grey values of all the pixels in the selection divided by the number of pixels to give a value of optical intensity (**Figure 2-6**).

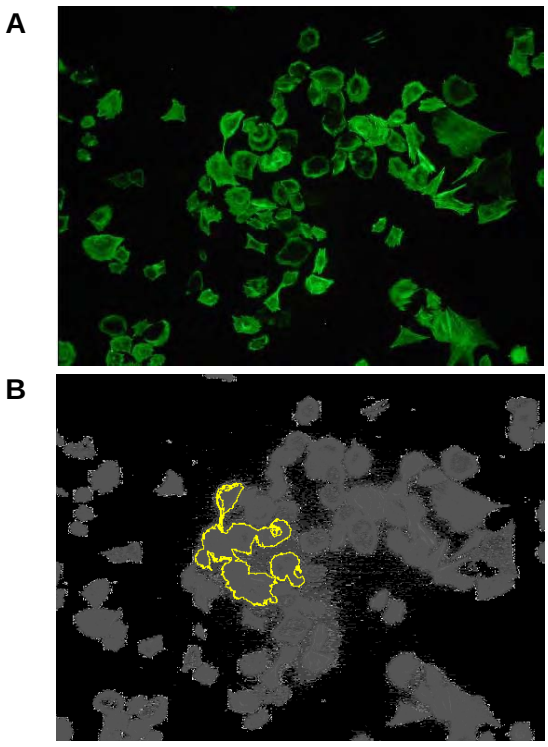


Figure 2-6 Quantification of actin fluorescent intensity stained with Alexa Fluor 488-phalloidin. Fluorescent intensity of the stained cells were measured using ImageJ by converting the (**A**) original fluorescent photomicrograph image to a (**B**) HSB stack that highlights different intensities of fluorescence in shades of grey. 100 grey areas were then highlighted (in yellow) using ImageJ tools to measure average pixel intensity to determine mean actin intensity.

2.4 ASSESSMENT OF CELL MORPHOLOGY AFTER US EXPOSURE

Cell lines were visualised immediately after a 30 minute US exposure (refer to **Section 2.2.2.2**) using an Eclipse TE300 phase contrast light microscope. Photomicrographs were captured as previously described in **Section 2.3.2** and subsequently uploaded to ImageJ software. Images were initially normalised by subtracting a background image from the original image before cell morphology and cell size were assessed. Cell size was obtained using the drawing tool in ImageJ to manually outline 100 cells per image to acquire average cell area in pixels. To convert pixels to area (μm^2), a photomicrograph was taken of a stage micrometer (E.Leitz, Wetzlar) at the same magnification as the original photomicrograph. Using ImageJ, the scale was set by measuring a known distance on the image of the micrometer then tracing a line on the original image to give the length of the line in pixels. By calculating the ratio of pixels to length, a global scale bar was then set for all images of each specific magnification. The scale was reset for each magnification used.

2.5 SCRATCH WOUND ANALYSIS

The scratch wound assay (Liang *et al.* 2007) was used to measure *in vitro* US-stimulated scratch wound closure rate. Prior to cell seeding, reference lines for plate orientation were scored onto the underside of a 6-well plate to ensure that images of the same scratch area were analysed at each timepoint. Using a disposable scalpel (Swann-Morton, UK), three horizontal lines were scored on the underside of each

well indicating the top, middle and bottom of the scratch. Three indentations were then etched on each horizontal line to indicate locations for 'scratch' indention (**Figure 2-7**). Cell lines were subsequently seeded at a density of 400,000 cells/well in supplemented DMEM and incubated at 37°C for 12 hours. Cultures were exposed to US delivered using the DuoSon as described in **Section 2.2.2.2.**

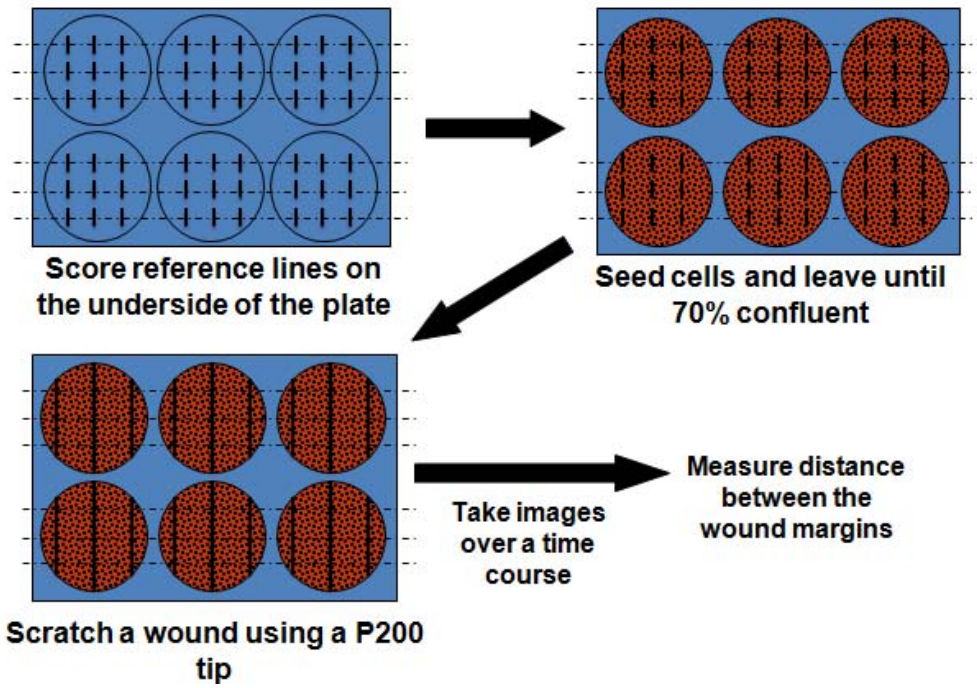


Figure 2-7 Schematic diagram of the scratch wound assay

After US exposure, supplemented DMEM was aspirated from each well and using the etched guidelines, vertical scratches were made in the US-stimulated cell monolayer using a p200 tip. The wells were subsequently washed with DMEM without supplements to remove cell debris before being replenished with

supplemented DMEM. Baseline photomicrographs of the scratches were captured immediately after US exposure and at each 24 hour interval until complete closure of the scratch wounds was confirmed. To ensure the identical location of the scratch at each timepoint was studied, the orientation/reference lines and notches were positioned at either the top or the bottom of each field before acquiring the image. Supplemented DMEM was replenished every 24 hours.

To identify the contribution of US-induced cell migration to scratch wound closure, parallel scratch wound experiments were performed in the presence of mitomycin C to (Sigma Aldrich, UK) to inhibit cell proliferation the scratch wound assay (Draper *et al.* 2003). After scratch wounds were introduced to the US-exposed monolayers as described above, the cells were washed and the medium was replaced with supplemented DMEM containing 0.05µg/ml of mitomycin C. Supplemented DMEM containing 0.05µg/ml of mitomycin C was replenished every 24 hours. Photomicrographs of the scratches were captured every 24 hours as described above.

2.5.1 DETERMINING RATE OF SCRATCH CLOSURE BY IMAGEJ ANALYSIS

All photomicrographs were imported into ImageJ software and scratch wound measurements were analysed by using a specially designed macro for the scratch wound assay (Landini G, 2010). The macro created definitive borders along the

wound edge that was then used to calculate the width in pixels of the denuded area from one edge of the scratch to the other at the widest point (**Figure 2-8**).

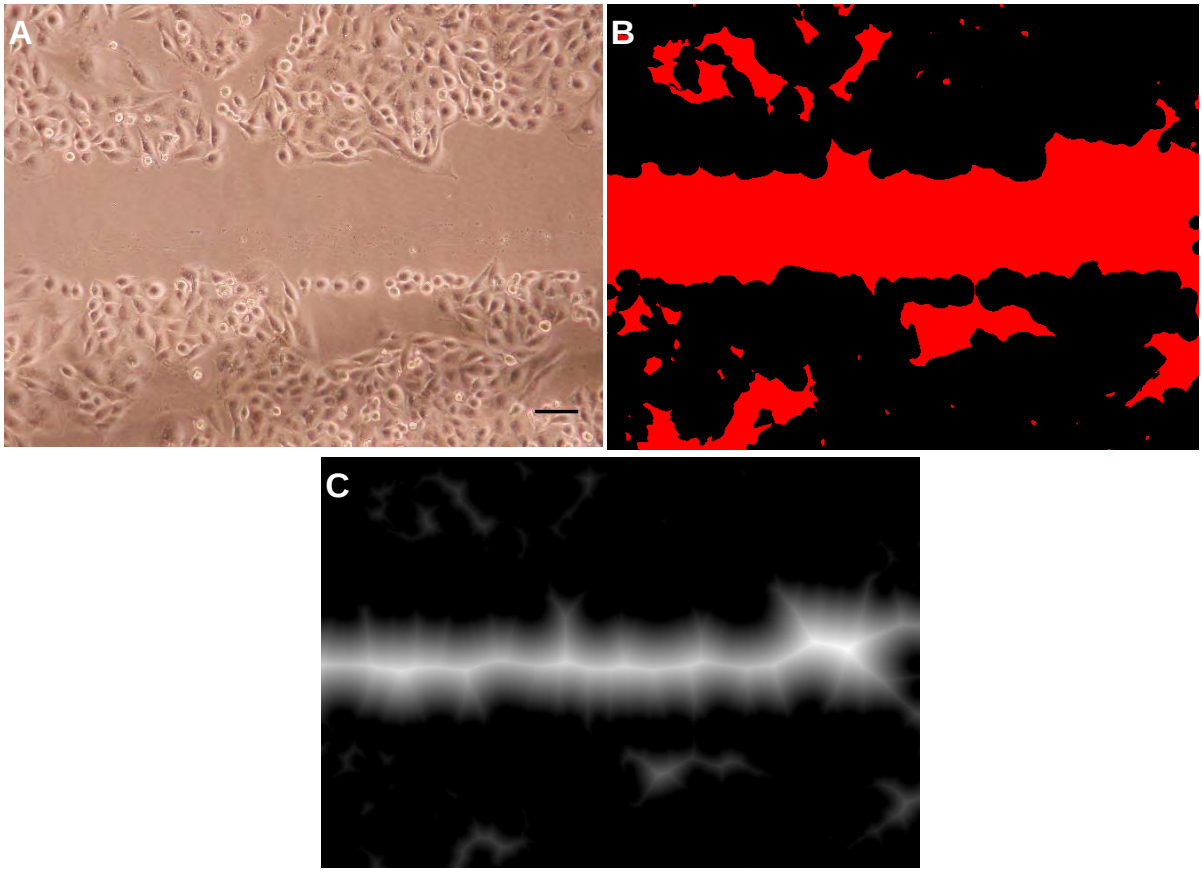


Figure 2-8 ImageJ analysis of the scratch wound assay using a custom-built macro. (**A**) Photomicrographs were taken of scratch wounds daily and subsequently uploaded to Image J to measure the rate of scratch wound closure. (**B**) Briefly, cell borders were outlined and (**C**) the denuded area of the scratch was highlighted. The width at the widest point of the denuded area was subsequently used to calculate the rate of scratch wound closure. Bar = 100μm.

The wound width in pixels was converted to wound width in μm^2 by dividing pixels by the number of pixels in 1µm. The number of pixels per µm was determined by setting the scale bar using ImageJ previously described in **Section 2.3.2.1**. The rate of scratch closure per hour was then calculated by dividing the timepoint at which the photomicrograph was taken by the distance closed, which is the width of the scratch at the timepoint the photomicrograph was taken subtracted from the width of the baseline scratch. To account for the two healing fronts, the calculated value was then divided by two to give a final rate in µm per hour.

2.6 DETERMINATION OF MATRIX MINERALISATION IN MDPC-23 CELLS

2.6.1 CELL CULTURE

MDPC-23 cells were seeded in 6-well plates at a density of 70,000 cells per well in supplemented DMEM. The cells were allowed to adhere for 12 hours at 37°C and subsequently washed in sterile DMEM without supplements. The medium was then replaced with mineralisation medium (MM) consisting of supplemented DMEM growth containing 5mM beta-glycerophosphate disodium salt hydrate (β-GP, Sigma-Aldrich, UK), 50µg/ml ascorbic acid (AA, Sigma-Aldrich, UK) and 10nM dexamethasone (dex, Sigma-Aldrich, UK). The cells were cultured in MM for 24 hours prior to US exposure and medium was replenished every 24 hours thereafter and prepared fresh on the day of use.

2.6.2 STAINING FOR MATRIX MINERAL USING ALIZARIN RED S DYE

Alizarin red S staining (ARS) was used to evaluate mineral deposition in MDPC-23 cell cultures exposed to US (Gregory *et al.* 2004). To assess for mineral deposition, formalin-fixed, US-stimulated MDPC-23 cells were washed twice with distilled water and the cells were left to air-dry. Meanwhile, a 40mM solution of ARS dye (BDH, UK) was prepared and pH adjusted to 4.2 using 10% acetic acid (Sigma-Aldrich, UK). The solution was then added to the cells at 1ml/well and gently agitated on a rotary shaker at room temperature for 20 minutes. Unincorporated ARS dye was gently aspirated from each well and the cells were washed with distilled water 4 times whilst being agitated for 5 minutes for each wash. The plates were then air-dried at room temperature to remove excess water.

Recovery and quantification of ARS dye was achieved by acetic acid extraction and neutralisation with ammonium hydroxide followed by colorimetric detection (Gregory *et al.* 2004). Briefly, stained cells were incubated in 10% acetic acid at room temperature for 30 minutes with gentle agitation. Cells were then detached from the culture well plastic using a cell scraper and the slurry from each well was transferred into individual 1.5ml Eppendorfs (Appleton Woods, UK). The tubes were vortexed for 30 seconds before placing into a water bath preheated to 85°C for 20 minutes. Afterwards, the tubes were immediately quenched on ice for 5 minutes, spun at 13,000rpm for 15 minutes and the supernatant transferred to a new Eppendorf. For every 150µl of supernatant, 45µl of 10% ammonium hydroxide (Sigma-Aldrich, UK)

was added to neutralise the acid in the solution. The absorbance of the extracted dye was then analysed using a spectrophotometer microplate reader at 450nm.

The concentration of incorporated ARS dye was determined according to an ARS dye standard curve. Briefly, a 30μM ARS dye solution was prepared and a concentration gradient was produced by serial dilution. Each concentration standard was adjusted to pH4.2 with 10% acetic acid or 10% ammonium hydroxide (Sigma-Aldrich, UK) before the absorbance of each standard was measured using a spectrophotometer microplate reader at 450nm. A standard curve was plotted using linear regression analysis resulting in the generation of a 3-parameter polynomial equation (**Figure 2-9**). Interpolation for the concentration of incorporated ARS dye was achieved by solving the polynomial equation. To determine mineral production in relation to cell number, a parallel experiment was performed to determine cell number in US-stimulated MPDC-23 cells cultured in MM. Cell number was assessed as described in **Section 2.2.2.1** at the same timepoint mineral deposition was determined by alizarin red S dye. Optical density was corrected for background absorbance by subtracting the optical density of MM or DMEM as well as ARS dye from the optical density of experimental and control controls, which was subsequently normalised to cell number to allow comparison of mineral content between groups.

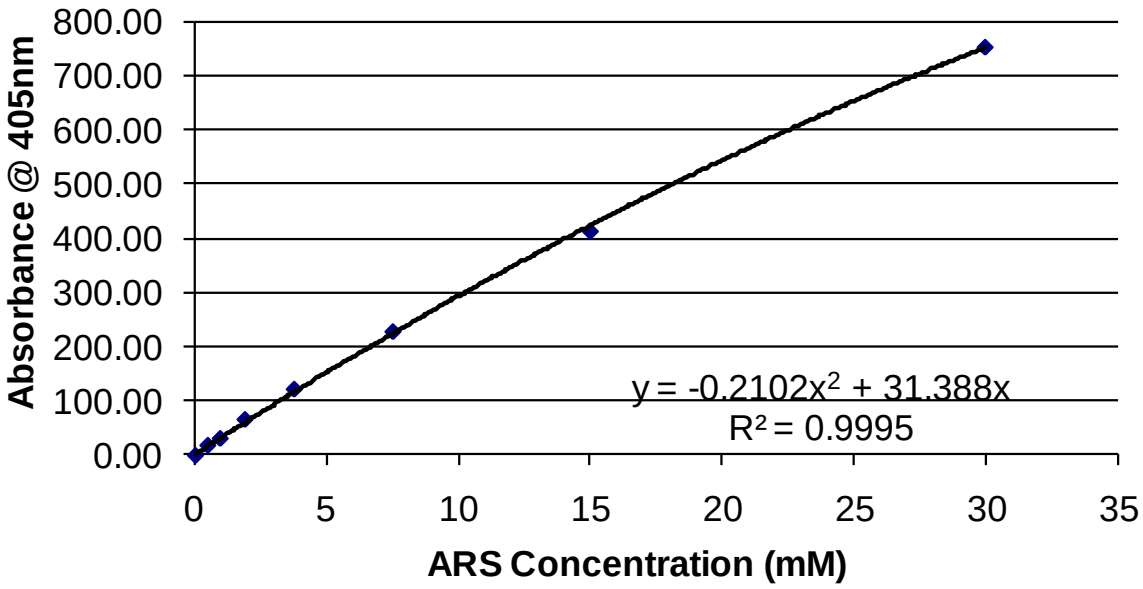


Figure 2-9 Quantification of Alizarin Red S Dye (ARS) to determine amount of mineral deposition. The amount of ARS was determined according to standard curve and was normalised to cell number to obtain the amount of mineral deposition.

2.7 GENE EXPRESSION ANALYSIS

2.7.1 PREPARATION OF US-EXPOSED CELLS FOR GENE EXPRESSION ANALYSIS

2.7.1.1 Dental Ultrasonic Scalers

A homogenous single cell suspension of MDPC-23 cells in supplemented DMEM was pipetted into a 5ml bijou at a density of 350,000 cells/ml and subsequently stimulated with low frequency US as described in **Section 2.2.2.1**. Ultrasound-stimulated cells were seeded into T25 flasks for 24 hours before RNA was isolated

for subsequent gene expression anaylsis by reverse transcription polymerase chain reaction (RT-PCR).

2.7.1.2 DuoSon

MDPC-23 cells were seeded in 6-well plates at a density of 70,000 cells per well in supplemented DMEM and left to adhere for 12 hours at 37°C. The cells were then washed with DMEM without supplements and the medium was replaced with MM (refer to **Section 2.6.1**). The cells were cultured in MM for 24 hours and subsequently stimulated with 45kHz US for 30 minutes as described in **Section 2.2.2.2**. RNA was isolated 12 days after initial US exposure for gene expression anaylsis by RT-PCR.

2.7.2 RNA ISOLATION AND RT-PCR

2.7.2.1 RNA isolation

Total RNA was extracted from US-stimulated MDPC-23 cells (refer to **Section 2.2.2.2**) using RNeasy (RNeasy mini kit, Qiagen, UK) in accordance with manufacturer's instructions. Briefly, US-stimulated MDPC-23 cells stimulated cells were collected by incubating in 350µl of buffer RT (supplied in kit) with 10µl beta-mercaptoethanol (β-ME, Sigma-Aldrich, UK) for 5 minutes at room temperature with agitation. The lysate was then transferred to a 1.5ml Eppendorf (Appleton Woods, UK) and mixed by rapidly pipetting the mixture. An equal volume of ice-cold 70% ethanol was added to the mixture and the lysate mixture was transferred to a spin

column supplied within the kit. Total RNA binds to the membrane of the column and removal of genomic DNA was achieved by adding DNase I (RNase-free DNase kit, Qiagen, UK) to the spin column and incubating for 15 minutes in accordance with the manufacturer's instructions. Contaminants were removed by a series of washes and elution steps by centrifugation and purified RNA was then eluted in RNase-free water. RNA concentration was quantified using a Biophotometer (Eppendorf, UK) at 260nm and RNA purity was assessed from the associated absorbance ratios at 260nm and 280nm, which was further confirmed by agarose gel electrophoresis (**Figure 2-10**)(**Section 2.7.2.5**).

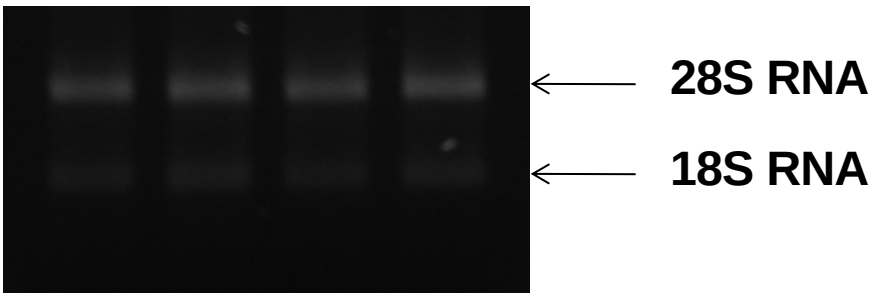


Figure 2-10 Total RNA from US-stimulated MDPC-23 cells isolated using the RNeasy mini kit. Ethidium bromide staining of rRNA reveals RNA integrity by electrophoresis on a 1% agarose gel.

2.7.2.2 Reverse Transcription

Single strand cDNA was synthesised using the Omniscript Reverse Transcriptase kit (Qiagen, UK) according to the manufacturer's instructions. Briefly, 1-2µg of cellular RNA (refer to **Section 2.7.2.1**) was added to 1µM of oligo(dT) primer (Ambion, UK)

for reverse transcription (RT). RNA-free, molecular grade water was then added to the mixture to give a total volume of the mixture of 14µl. This was then incubated in a waterbath preheated to 80°C for 10 minutes to allow the mRNA template to anneal to the oligo(dT) primer and the reaction is subsequently stopped by quenching the sample on ice for 5 minutes. To the mixture, 1x buffer RT (supplied within the kit), 0.5mM dNTP mix (supplied within the kit), and 10U RNase inhibitor (supplied within the kit) was added, briefly vortexed and briefly centrifuged to collect residual liquid. The reaction mixture was incubated at 37°C for 60 minutes for primer extension and subsequent complementary nucleotide synthesis thereby creating an mRNA/cDNA hybrid. To synthesise a complementary DNA strand, the template RNA was digested by incubating the sample at 95°C for 5 minutes.

Following incubation, the cDNA sample was concentrated using the Microcon centrifugal filter device (Millipore, UK). Briefly, the cDNA sample was made up to a total volume of 500µl with RNA-free water and transferred by pipetted into a Microcon filter device. The filter was then spun at 10,000rpm for 6.5 minutes and the eluant was subsequently discarded. A further 500µl of RNA-free water was added to each filter device and centrifuged at 10,000rpm for 7 minutes and the eluant was once again discarded. The volume of liquid remaining in the filter device was then measured and if the volume exceeded 50µl, the filter was centrifuged at 10,000rpm for 30 seconds intervals until the volume measured 50µl or below. Upon reaching the final volume, the filter was inverted into a 1.5ml collection tube (Millipore, UK) for cDNA collection by centrifugation at 4000rpm for 1 minute. Single strand cDNA was

quantified using a Biophotometer at 260nm and purity was assessed from the associated absorbance ratios at 260nm and 280nm. The cDNA samples were then stored at -20°C until required.

2.7.2.3 Polymerase Chain Reaction (PCR)

2.7.2.3.1 Oligonucleotide Primers

All oligonucleotide primers were specific to mouse species and custom synthesised by Invitrogen, UK (see **Table 5**). The lyophilised primers were dissolved in sterile water to a final concentration of 100µM for use. These stocks were further diluted with RNA-free water to provide a 25µM working stock solution and stored at -20°C.

Table 5 Primer sequences with corresponding annealing temperatures, cycle numbers and expected product size used for RT-PCR

Gene	Abbreviation	Product Size (bp)	Annealing Temperature (°C)	Cycle Number	Primer Sequence
Alkaline Phosphatase	ALP	501	61.5	30-35	5'-CCAGCAGGTTTCTCTCTTGG-3' Sense 5'-GGGGAGTGTGTGTGTG-3' Anti-sense
Bone Morphogenetic Protein 2	BMP2	396	60.5	28	5'- TGGAAGTGGCCCATTTAGAG-3' Sense 5'-CATGCCTTAGGGATTTGGA-3' Anti-sense
Bone Morphogenetic Protein 4	BMP4	369	60	25	5'- CAGGGCTTCCACCGTATAAA-3' Sense 5'-ATGCTTGGGACTACGTTTGG-3' Anti-sense
Myelocytomatosis Oncogene	c-myc	324	60	30-35	5'-TCTCCACTCACCAGACAAC-3' Sense 5'-TCGTCTGCTTGAATGGACAG-3' Anti-sense
Collagen, type I, alpha 1	COL1A1	499	60	27	5'- AAAAGGGTCATCGTGGCTTC-3' Sense 5'-ACTCTGCGCTCTTCCAGTCA-3' Anti-sense
Dentine Matrix Acidic Phosphoprotein 1	DMP1	300	58	31	5'- CAGGACAGGGAGTGACCAGT-3' Sense 5'-CCCCACCAAATTTTCTTCCT-3' Anti-sense
Dentine Sialophosphoprotein	DSPP	598	56	22	5'- GGA TCA TCA GCC AGT CAG-3' Sense 5'-GTC ATG GTC ACT GCT GTC-3' Anti-sense
Glyceraldehyde 3-Phosphate Dehydrogenase	GAPDH	450	60.5	23	5'- CCCATCACCATCTTCCAGGAGC-3' Sense 5'-CCAGTGAGCTTCCCGTTCAGC-3' Anti-sense
Heat Shock 27kDa Protein	HSPB	393	60	37	5'- GGC TCA GTG AAG GCA AGT TC-3' Sense 5'-GGG AGG TAG ACA GGG GAT TC-3' Anti-sense
Heat Shock 70kDa Protein	HSPA	362	60	37	5'- CGA CCT GAA CAA GAG CAT CA-3' Sense 5'-CCAA GG TCA CCT CGATCT GT-3' Anti-sense
Nestin	NES	445	60.5	31	5'- AGG TGT CAA GGT CCA GGA TG-3' Sense 5'-GGC CTA GAT GCA CAG GAG AC-3' Anti-sense
Osteoadherin	OMD	395	60	27	5'- CGA CCT GAA CAA GAG CAT CA-3' Sense 5'-ACTTGCAGGGCAGAGAGAGA-3' Anti-sense
Osteocalcin	BGLAP	479	61.5	27	5'- AAGCAGGAGGGCAATAAGGT-3' Sense 5'-ACTTGCAGGGCAGAGAGAGA-3' Anti-sense
Transforming Growth Factor Beta 1	TGFB1	438	60	27	5'- CTGTCCAAACTAAGGCTCGC-3' Sense 5'-CGTCAAAAGACAGCCACTCA-3' Anti-sense
Vascular Endothelial Growth Factor	VEGF	431	55	37	5'- CTGCTCTCTTGGGTCCACTGG-3' Sense 5'-TACACTGTTCGGTTGGGCCAC-3' Anti-sense
FMS-like Tyrosine Kinase 1	FLT1	505	62	50	5'-TGTGGAGAAACTTGGTGACCT -3' Sense 5'-TGGAGAACAGCAGGACTCCTT-3' Anti-sense
Kinase Insert Domain Protein Receptor	KDR	382	58	50	5'-AGAACACCAAAAGAGAGGAACG -3' Sense 5'-GCACACAGGCAGAAACCAGTAG-3' Anti-sense

2.7.2.3.2 PCR Set-up and Cycling Conditions

PCR was performed using REDTaq® ReadyMix (Sigma-Aldrich, UK) as per the manufacturer's instructions. All reagents and reactions were kept on ice to prevent degradation of cDNA and primer products and premature non-specific amplification. For each set of primers, the following mastermix was prepared in a 0.2ml PCR tube (Appleton Woods, UK) (**Table 6**):

Table 6 PCR mastermix prepared for each reaction.

Reagent	Volume (µl)	Final Concentration
REDTaq® ReadyMix™	12.5	1.5 Units Taq DNA
Forward Primer	0.5	25mM
Rnase Inhibitor	0.5	25mM
cDNA	<2	50ng
Rnase Free Water	25-all of above	n/a

50ng of cDNA (see **Section 2.7.2.2**) was added to the mastermix containing the gene of interest. The samples were gently agitated and briefly centrifuged to collect residual liquid and subsequently transferred to a Mastercycler® Gradient Thermal Cycler (Eppendorf, UK) for amplification. Activation of REDTaq® was achieved by heating the samples to 95°C for 5 minutes prior to thermal cycling (refer to **Table 7**).

Table 7 Thermal cycler conditions per reaction for the Mastercycler® Gradient Thermal Cycler

Cycling Condition	Temperature (°C)	Time (secs)
Denaturation	95	20
Annealing	Primer specific	20
Extension	72	20

After the designated number of cycles, 6µl of the amplified product was then used for visualisation and quantification using gel electrophoresis (**Section 2.7.2.5**). To account for inter-sample variation, PCR products were normalised using the house-keeping gene glyceraldehydes-3-phosphate-dehydrogenase (GAPDH) as an internal control (Robbins and McKinney 1992).

2.7.2.4 Agarose Gel Electrophoresis

For RNA visualisation, a 1% non-denaturing agarose gel was prepared using 0.6g of agarose (Web Scientific, UK) dissolved in 60ml of 1x TAE buffer, a mixture of 2M Tris-acetate and 0.05M EDTA (Qiagen, UK), by heating the suspension in a microwave (Sanyo, UK). To visualise PCR products, a 1.5% agarose gel was made using 0.8g of agarose dissolved in 60ml of 1x TAE buffer by mixing and heating in the same manner as described above. The subsequent molten agarose was cooled to approximately 50-55°C under running cold water before the addition of ethidium bromide (Helena Biosciences, UK) at a concentration of 10mg/ml. The mixture was then gently mixed and poured in a casting tray. Combs were added to the tray to

create loading chambers/wells before the agarose was left to solidify at room temperature. Once solidified, the gel was immersed in an electrophoresis tank filled with 1x TAE buffer and the well forming combs removed.

To enable visualisation of RNA, a 5x loading buffer (VWR, UK) was added to the samples before transferring 6µl of the sample into each well. Amplified PCR products were loaded directly into the wells at the same volume and the gels were run at 120 volts (V) for 35 minutes until separation of nucleic acids was achieved by electrophoresis. For reference, Hyperladder IV (Bioline, UK) was run alongside the samples of each gel to enable accurate sizing of the PCR products.

2.7.2.5 Gel Visualisation and Quantification

Following electrophoresis, the gel was transferred to the G:BOX (Syngene, UK) gel imaging system for image capture that was subsequently uploaded to GeneSnap image acquisition software (Syngene, UK) for quantification. To obtain the volume density of amplified products, the band intensity of the gene of interest was normalised against the band intensity of GAPDH. Normalised values were expressed as a percentage of the highest volume density value obtained.

2.8 STATISTICAL ANALYSIS

All statistical analyses were performed using Microsoft Excel™ 2007 data analysis toolpak. Statistical significance was analysed using one-way ANOVA by comparing between two values. Unless stated otherwise, all values were expressed as the mean ± standard deviation (SD) and replicates (*n* numbers) were given in the accompanying text. A value of $P<0.05$ was considered to be significant and were denoted as * ($P<0.05$), ** ($P<0.01$) and *** ($P<0.001$) in table and figures.

CHAPTER 3 RESULTS

EFFECT OF LOW FREQUENCY ULTRASOUND EMITTED BY DENTAL ULTRASONIC SCALERS ON ODONTOBLAST-LIKE CELLS

The dental ultrasonic scaler are valuable instruments for the prevention of periodontal disease and these devices and associated scaler tips have been extensively assessed for their ability to effectively remove calculus, alter the topography of the tooth surface and for debriding periodontally involved root surfaces (Gankerseer and Walmsley 1987; Jacobson *et al.* 1994; Lee *et al.* 1996; Brine *et al.* 2000). Increased efficiency of these instruments for treating periodontal disease is accompanied by undesired biological effects, including damage to pulp vitality and increased tooth sensitivity due to loss of dentine as a consequence of exposure of dentinal tubules (Atrizadeh *et al.* 1971; Fischer *et al.* 1991). Research into the cellular effects of treated tissues using ultrasonic scalers is minimal. However, the beneficial effects of US observed in treatment of bone fractures and in soft tissue healing (Zhou *et al.* 2004; Claes and Willie 2007) may also apply to oral tissues exposed to ultrasonic dental scalers (Scheven *et al.* 2009b). Characterisation of US-mediated events may subsequently lead to development of improved instrument designs for clinical application whilst minimising damage to biological tissues. The aims of the present study were two-fold:

1. To determined if the use of clinically applied US may have therapeutic value dentally.

2. Identification of appropriate US *in vitro* delivery conditions and how the conditions applied may affect cellular behaviour by characterisation of VEGF and Wnt signalling pathways.

3.1 THERMAL EFFECTS BY ULTRASONIC SCALER TIP

Dental ultrasonic scalers produce an increase in temperature at the tooth surface due to frictional heating with contact between scaler and tooth, direct temperature application by the irrigation fluid and acoustic energy absorption of US transmitted into the tooth (Trenter and Walmsley 2003). Temperature rises in the tooth caused by heating can cause damage to the pulp and dentine resulting in irreversible pulpitis (Wilder-Smith *et al.* 1998) and tissue necrosis (Nyborg and Brannstrom 1968). Temperature rise using the analytic D-tip scaler probe was therefore measured for each chosen scaler tip to determine potential damage to exposed cells *in vitro*.

A small and steady rise in temperature was observed during US application by all three ultrasonic scaler tips applied over a period of 5 minutes. Temperature rise was calculated by subtracting temperature measured at baseline from the recorded temperature after 5 minutes of US exposure. A variation in initial medium temperature was observed due to fluctuations in room temperature. At the highest power setting (P7), there was less than 1°C temperature rise within the medium using all tip designs. The Angled tip (**Figure 3-1A**) produced the greatest rise in temperature for all 3 power settings applied resulting in temperature rises of 0.8°C, 0.9°C and 0.9°C at power settings 1, 5 and 7, respectively.

Both the 076 tip and the D-tip produced similar temperature profiles compared with those of the Angled tip where the highest power setting generated the greatest temperature rise (**Figure 3-1**). Both the 076 tip and the D-tip scaler tips resulted in

lower temperature rises in medium compared with the Angled tip whilst the D-tip produced temperature rises lower than the Angled tip. The 076 tip increased the temperature of the growth medium by 0.5°C at P1, 0.7°C at P5 and 0.7°C at P7 whereas the D-tip raised the temperature of growth medium by 0.4°C, 0.5°C and 0.7°C at P1, P5 and P7, respectively (**Figure 3-1B, C**).

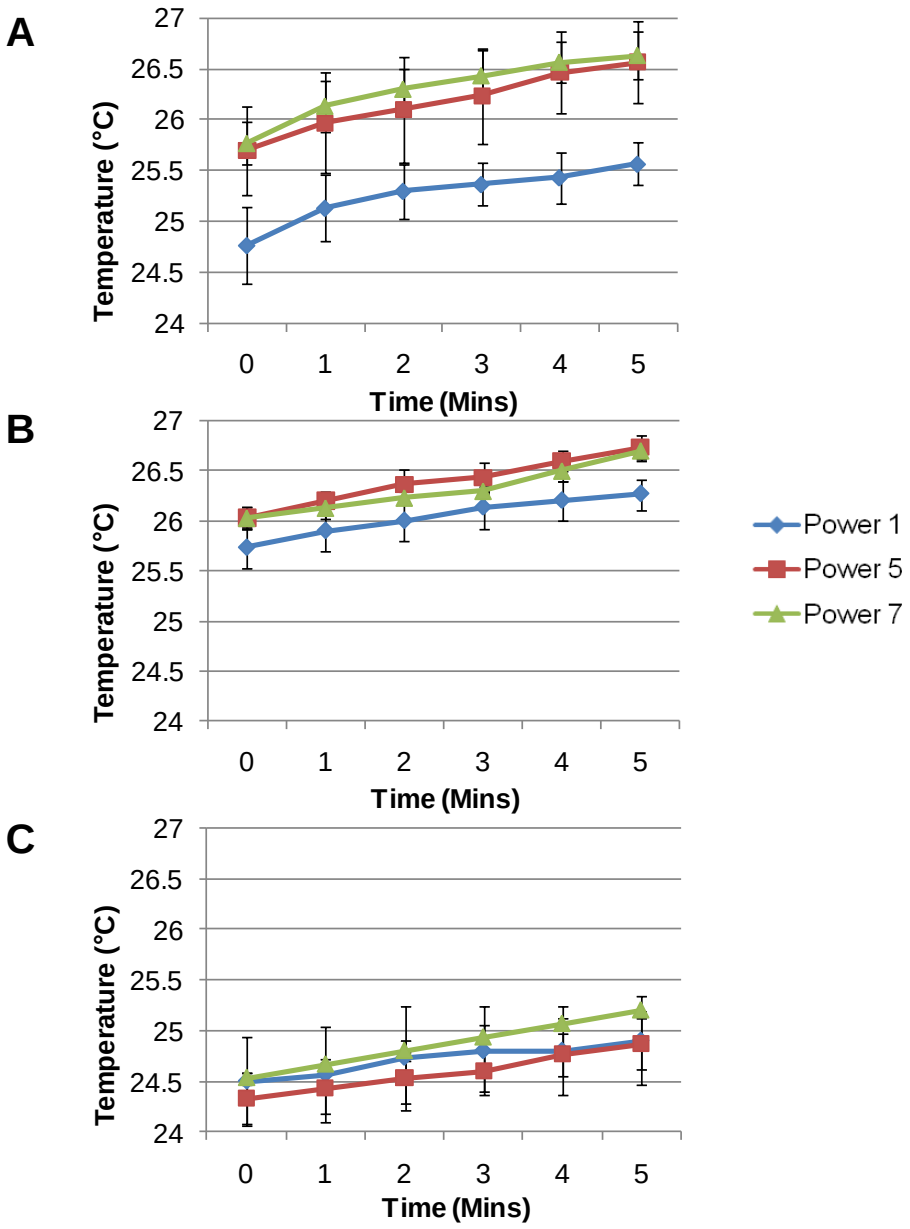


Figure 3-1 Temperature rise of cell culture medium during 5 minutes of US application by (**A**) the Angled dental tip, (**B**) 076 tip and (**C**) the D dental tip at three incremental power settings (P1, P5 and P7). Temperature rise in medium was power dependent and the highest temperature rise was generated using the Angled Tip with the D-tip producing the lowest thermal effect in cell culture medium. Actual temperatures are plotted for each power setting which was measured in triplicate and data is expressed as mean temperature of 3 experiments ±SD.

3.1.1 CELL VIABILITY FOLLOWING ULTRASOUND EXPOSURE

Cell viability was determined after 5 minute of US exposure immediately using trypan blue (**Section 2.2.2.1**). Different cell viability profiles were observed when MDPC-23 cells were exposed to the three ultrasonic scaler tips (**Figure 3-2**). The 078 tip did not significantly affect cell viability whereas the Angled tip and the D-tip reduced viable MDPC-23 cell numbers. The greatest decrease in viable cells was observed following exposure to the D-tip and generated a 16% decrease in viability at the highest power setting P10.

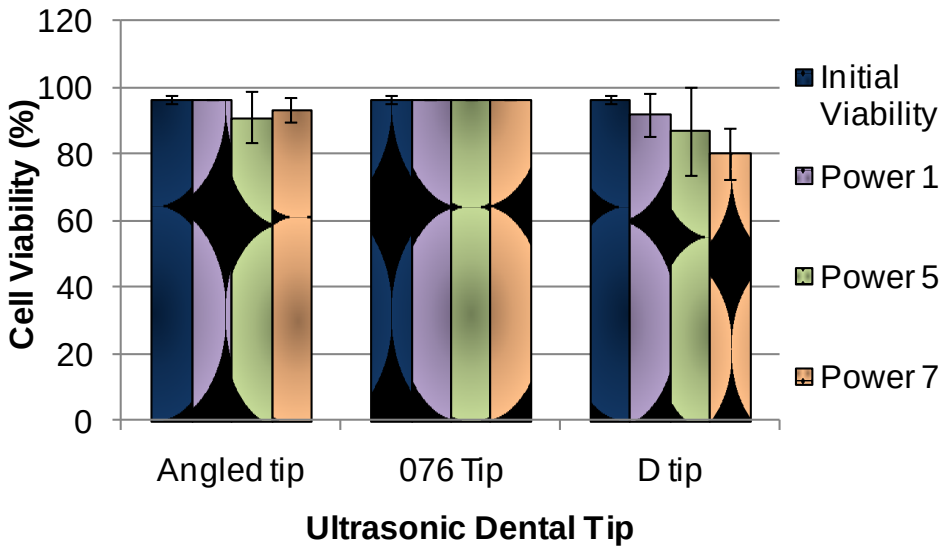


Figure 3-2 MDPC-23 cell viability following 5 minutes of US exposure using 3 dental scaler tips at three incremental powers. A power-dependent decrease in viability was observed in the D-tip whereas a loss in cell viability using the Angled tip was observed only at higher powers and cell viability was not affected using the 076 tip. No statistical difference was found when compared to control. The experiment was performed in triplicate and data is expressed as mean percentage viability of 3 experiments ±SD.

3.2 ROLE OF ULTRASOUND IN VEGF SIGNALLING

VEGF is expressed by odontoblasts and cells of the dental pulp during angiogenesis and reparative dentinogenesis (Telles *et al.* 2003; Grando Mattuella *et al.* 2007). Increased VEGF expression and angiogenesis has also been implicated in US-induced bone formation (Doan *et al.* 1999; Lu *et al.* 2008a). Enhanced expression of OPN, TGF-β1 and collagen type I, present during repair of mineralised tissues, were previously observed after US exposure using the analytic D-tip scaler probe (Scheven et al. 2007). Ultrasound may therefore stimulate reparative responses in the dentine-pulp complex by up-regulating VEGF expression (Scheven *et al*, 2009). The effect of US on VEGF expression was therefore investigated in odontoblast-like cell cultures.

$VEGF_{120}$ transcript levels were increased in MDPC-23 cells in a dose-dependent manner (**Figure 3-3A**) and $VEGF_{120}$ expression values were higher in MDPC-23 cells 24 hours after US exposure than in MDPC-23 cells 4 hours after US stimulation. MDPC-23 cells stimulated with US at P3 and P5 were ~75% higher in $VEGF_{120}$ expression 24 hours after exposure compared with 4 hour samples at the same powers (**Figure 3-3B**).

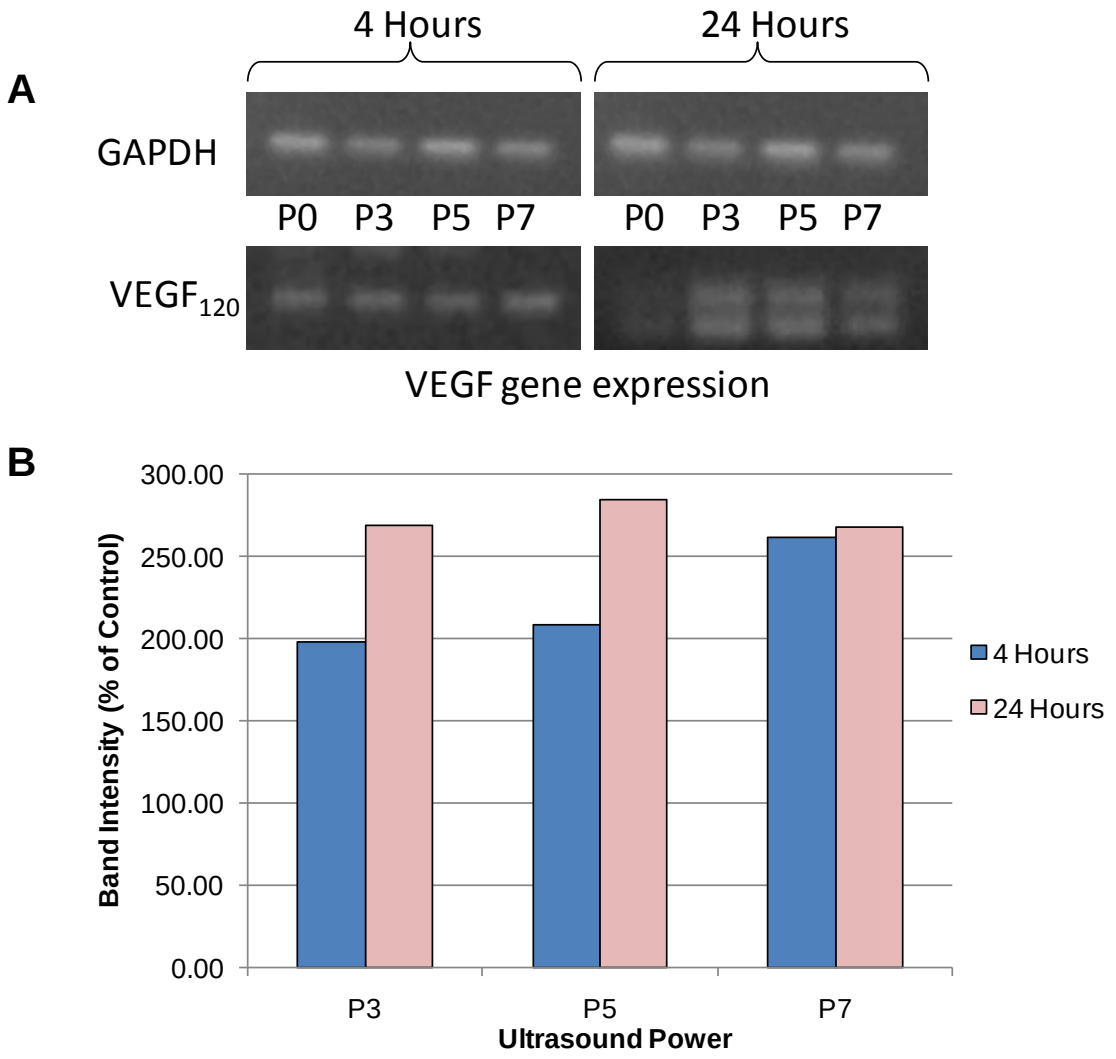


Figure 3-3 Effects of US on VEGF gene expression in MDPC-23 cells. (**A**) Representative gel images following RT-PCR analysis of GAPDH and $VEGF_{120}$ gene expression are shown at 4 hours and 24 hours post US exposure at three incremental powers settings. Ultrasound-induced VEGF expression was highest at all US power settings applied in 24 hours samples when compared to sham-treated control (P0). (**B**) Results of image analysis of RT-PCR products supported these findings with regards to VEGF expression at its highest 24 hours after US exposure at P5. The data was normalised to GAPDH expression and are shown as percentage of controls for one experiment (controls set to 100%).

To determine whether VEGF may act in an autocrine manner in MDPC-23 cells VEGF receptors 1 and 2 (VEGFR-1 and -2) transcript levels were analysed in this cell type. Data indicated that both VEGFR-1 and -2 were expressed in MDPC-23 cells (**Figure 3-4**).

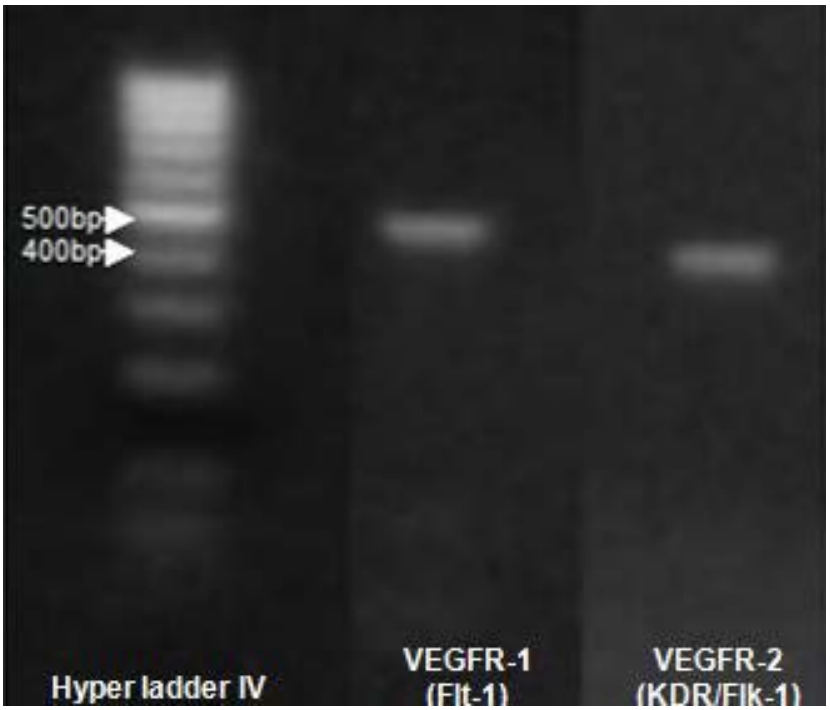


Figure 3-4 VEGFR-1 (Flt-1) and VEGFR-2 (Flk-1) as determined by RT-PCR analysis were detected in MDPC-23 cells. Hyperladder IV was used for comparison to determine product size for VEGFR-1 (505bp) and VEGFR-2 (382bp) amplified products.

To determine whether MDPC-23 cells responded to VEGF, cultures were incubated with rhVEGF for 48 hours at concentrations previously documented to stimulate cell proliferation. Following incubation, rhVEGF at 50 and 100ng/ml significantly

increased viable cell number compared with control cultures indicating stimulation in cell proliferation (**Figure 3-5**). Notably, 50ng/ml of rhVEGF has previously been demonstrated to stimulate rapid induction of human dental pulp cell proliferation (Matsushita *et al.* 2000) and increase microvascular density in tooth slice-based models (Goncalves *et al.* 2007).

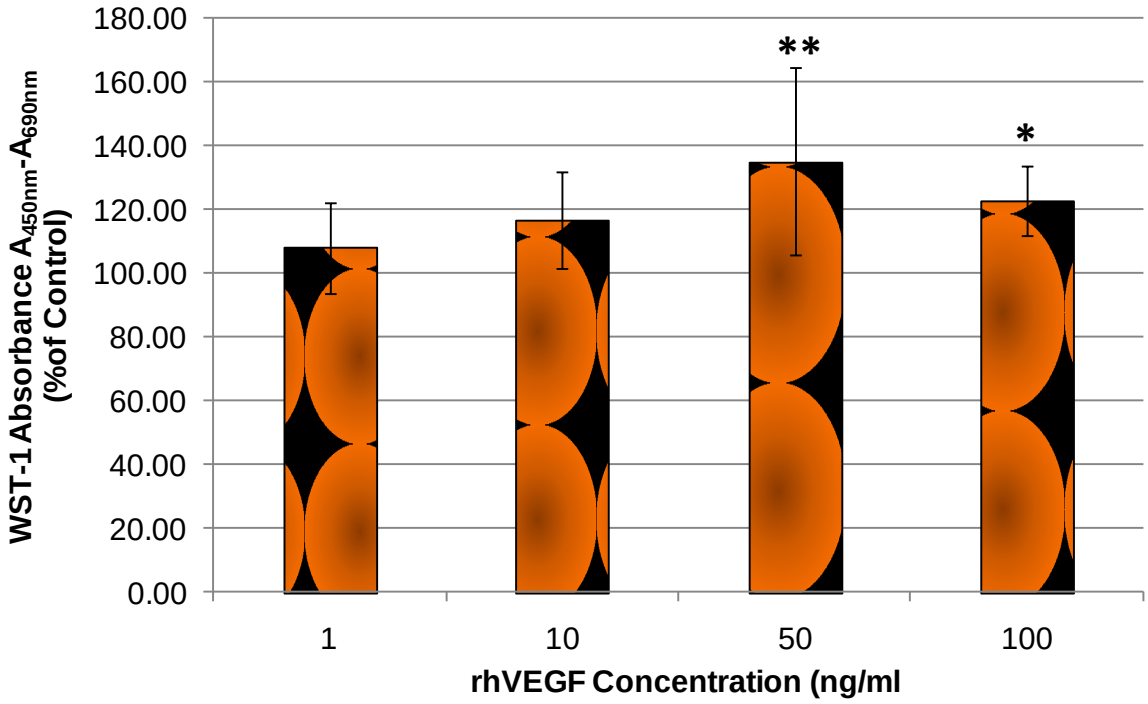


Figure 3-5 MDPC-23 cell numbers were significantly increased when cultures were exposed to in 50 and 100ng/ml of recombinant human (rh)$VEGF_{165}$ for 48 hours. Data analysis was by one-way ANOVA and values expressed as percentage of control from triplicate measurements of 3 experiments +/-SD. *P<0.05, **P<0.01 when compared with cultures without $rhVEGF_{165}$.

3.3 ANALYSIS OF WNT SIGNALLING FOLLOWING ULTRASOUND EXPOSURE

Both US and VEGF stimulation have previously been shown to activate the Wnt signalling cascade in several cell types with relevance to tissue repair and mineralised tissue biology (Dufourcq *et al.* 2002; Orlandini *et al.* 2003; Olkku *et al.* 2010). In addition, β-catenin activation has been associated with increased cell proliferation (Yano *et al.* 2005); (Takeuchi *et al.* 2008). Subsequently, changes to Wnt-signalling in MDPC-23 cells were investigated by immunocytochemical analysis of β-catenin (**Section 2.3.1**).

MDPC-23 cells exposed to US appeared to exhibit increased intracellular levels of β-catenin compared with controls. Nuclear accumulation of β-catenin, as identified by cells with more intensely stained nuclei, was apparently higher in MDPC-23 cells 24 hours post-US exposure compared with 4 hours after US treatment indicating US-induced Wnt/β-catenin activation occurred at a relatively late time point post-US treatment. Notably, increased gene expression in MDPC-23 cells was also previously detected 24 hours after exposure using a dental ultrasonic scaler (Scheven et al. 2007). Immunocytochemistry also indicated that whilst cytoplasmic β-catenin accumulation was evident in MDPC-23 cells 4 hour post US exposure, nuclear staining of β-catenin was more prominent in MDPC-23 cells 24 hours after US exposure (**Figure 3-6A**). Quantification using Image J analysis (**Section 2.3.2.1**) supported the immunocytochemical observations (**Figure 3-6B**).

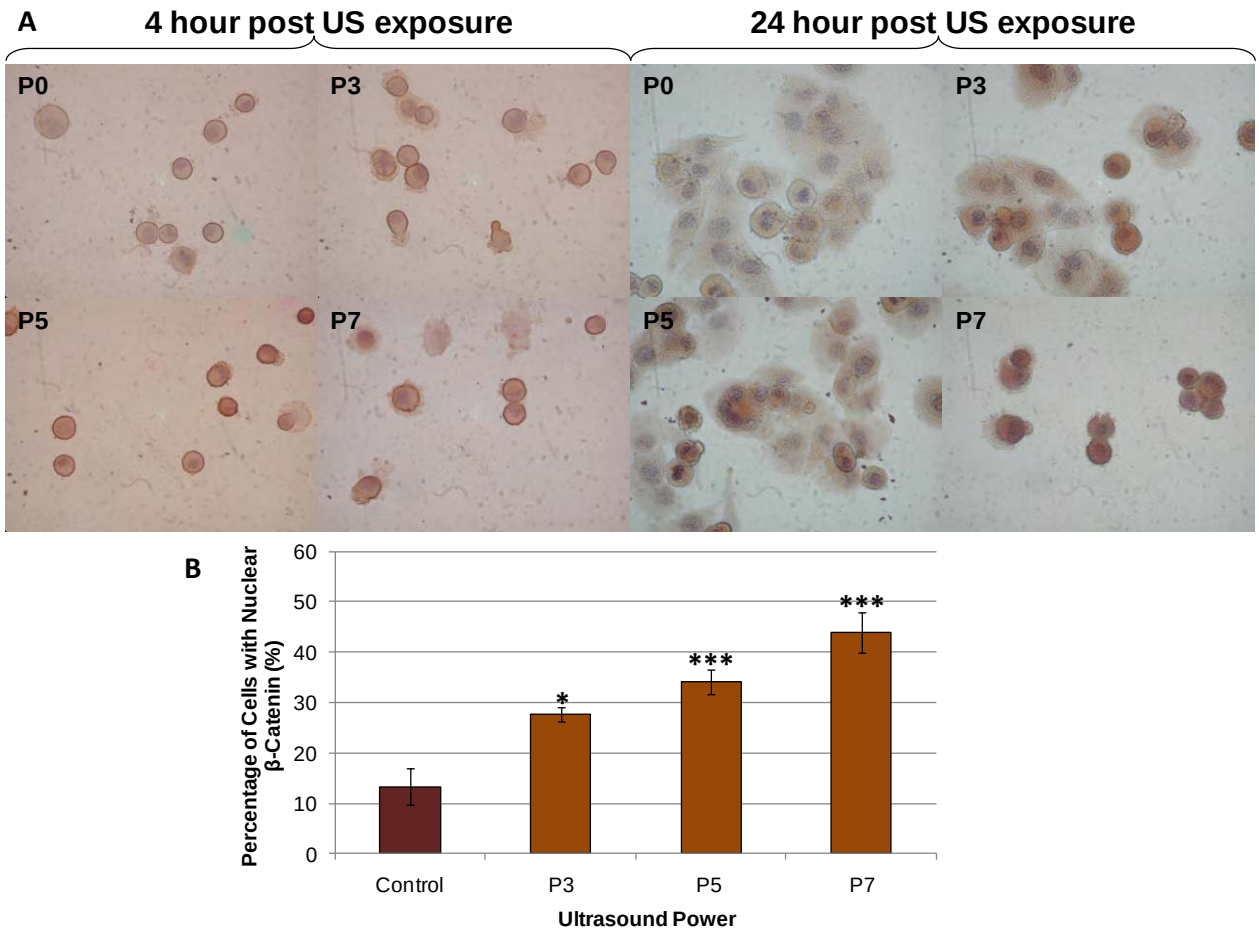


Figure 3-6 Immunocytochemical analysis of β-catenin staining in MDPC-23 cells 4 and 24 hours after US exposure at incremental US power settings (control, P3, P5, P7). (**A**) Light microscopy (LM) images showed increased nuclear staining of β-catenin in 24 hours post US samples at all US power settings when compared with control. Bar = 100μm. (**B**) Quantification of nuclear β-catenin staining in MDPC-23 cells 24 hours after US exposure using ImageJ analysis confirmed the appearance observed in the LM images and a significant dose-dependent response was observed. Data is expressed as mean positive nuclear staining as a percentage of total cell number counted for 3 independent experiments ± SD. *P<0.05, ***P<0.001 when compared with cultures in the absence of β-catenin antibody.

3.4 DISCUSSION

Low frequency US emitted by dental scalers produced minimal influence on temperature after 5 minutes of treatment. Moreover, low frequency US treatment of MDPC-23 cells resulted in limited cell death at the highest power setting applied. Culture temperature fluctuations may affect cell behaviour (Welgus *et al.* 1981; Flour *et al.* 1992). Heat stress induced at 42°C can affect cell viability by triggering a complex cascade of signaling events, including Ras, Rac1, mitogen-activated protein kinase (MAPK) and pro-survival factors (Park *et al.* 2005; Scheven et al. 2007). In fact, Scheven *et al.* (2007) demonstrated that low frequency US increased levels of heat shock protein (HSP) 25/70 transcripts following treatment of odontoblast-like cells. This present model for *in vitro* US delivery produced minimal temperature increases and it is concievable that US-stimulated biological effects were the result of mechanical stimulation of the cells by non-thermal cavitational and microstreaming.

An explanation for the differences observed in cell viability following application of the three tested dental scaler tips could relate to the transmission characteristics of each US tip insert. Notably a flatter, more rectangular-shaped ultrasonic tip was more effective at displacing water than a thinner, cylindrical tip (Felver *et al.* 2009). Indeed the D-tip had the largest and flattest surface area of the three ultrasonic tips tested so may have produced the greatest non-thermal effects on surrounding cells in suspension. The D-tip was selected for further studies into US-induced bioeffects as this tip produced a dose-dependent response between power and viability. Although the A tip and the 076 tip did not affect cell viability, it cannot be concluded that the

biology of the cells was not effected by tip displacement as amplitude varies between tip designs that ultimately may alter the acoustic power output (Lea *et al.* 2003). Ultrasound is emitted from a scaler tip at a range of points creating a figure of eight pattern surrounding the tip (Lea *et al.* 2009). The characteristics of the US wave changes due to scaler tip age and wear, load conditions and even how tightly the tip is inserted into the handpiece (Shah *et al.* 1994).

Low frequency US emitted through the D-tip was previously shown to have an effect on gene expression, adhesion as well as viability in MDPC-23 cells. Scheven *et al.* (2007) demonstrated that this scaler tip promoted a dose response for HSP25/7-expression as well as expression of TGF-β1, OPN and collagen type I, key markers of odontoblast differentiation, tooth development and repair. Interestingly low frequency US was able to increase VEGF transcript expression in MDPC-23 cells. Whilst VEGF is known to stimulate pro-angiogenic mechanisms within tissues, this molecule also has other non-vascular effects, including increasing cell proliferation via autocrine mechanisms (Lachgar *et al.* 1996; Thi *et al.* 2007). Notably, this study showed that VEGF increased MDPC-23 cell numbers, potentially via binding of VEGFR-1 and -2 on these cells. Interestingly, the data from this study corroborate previous findings by Matsushita *et al.* (2000) who demonstrated that VEGF increased human dental pulp cell proliferation and alkaline phosphatase levels via activation of these receptors (Matsushita *et al.* 2000). These experiments suggested that low frequency US has the potential to stimulate tooth repair by induction of VEGF involving cellular responses such as cell proliferation and neo-angiogenesis.

The specific pathways by which US delivers its cellular effects remains unclear. Activation of mechanoreceptors by US, such as integrins, may enable biomechanical force transduction within the cell or tissue (Ingber 1991) . Recent data has now implicated the Wnt/β-catenin pathway as a potential mediator for biomechanical and US signalling (Olkku *et al.* 2010). Furthermore, Wnt signalling has been found in numerous studies to be important in tooth and skeletal development (Gong *et al.* 2001; Krishnan *et al.* 2006; Scheller *et al.* 2008). Data presented here has demonstrated a dose-dependent response in β-catenin staining within 24 hours of low frequency US stimulation in the odontoblast-like cell line, MDPC-23 cells. The clear nuclear and cytoplasmic accumulation of β-catenin in US-stimulated cells shortly after exposure confirms activation of the canonical Wnt pathway. The range of intensities used in this present study ranged from 170mW/cm^2 to 920mW/cm^2 (Scheven et al. 2007) which corresponded with therapeutically accepted doses that have been demonstrated to stimulate mineralisation-associated events *in vitro* (Wiltink *et al.* 1995; Harle *et al.* 2001b; Harle *et al.* 2005) and *in vivo* (Dyson and Brookes 1983; Chang *et al.* 2002).

The data presented here indicate that non-thermal low frequency US emitted via a dental ultrasonic scaler has the ability to affect dental cell viability and increase the production of the angiogenic growth factor, VEGF. In addition, US may directly activate the Wnt/β-catenin pathway or mediate this molecular effect via an autocrine factor such as VEGF or TGFβ. The stimulation of VEGF via receptor signalling and increase in β-catenin are both regulated by the PI3-Akt pathway (Olsson *et al.* 2006; Takeuchi *et al.* 2008). This survival pathway is activated in various cells in response

to mechanical stress (Garcia-Cardena *et al.* 2000; Kim *et al.* 2002) and the phosphorylation of the downstream targets are likely to contribute to US-induced cell survival, growth and proliferation (Hill *et al.* 2005; Sena *et al.* 2005). This present study concluded that low frequency US delivery had no major impact on MDPC-23 cell viability; however, this does not necessarily imply absence of cell injury and may represent post-US cell survival by mechanisms such as the PI3-Akt pathway.

CHAPTER 4 RESULTS

IN VITRO APPLICATION OF A CALIBRATED THERAPEUTIC ULTRASOUND DEVICE

Low frequency US emitted by dental ultrasonic scalers were assessed for their biological effects as described in Chapter 3, however, there were limitations in the experimental design in particular the precise US transmission and intensity were unknown. To investigate how US affects cellular function and to better understand the complex mechanisms by which US may exert these effects, a highly-controlled, well-characterised model for *in vitro* US delivery was required. While accelerated osteogenesis by exposure to LIPUS is reported in both animal models and in clinical trials (Duarte 1983; Klug *et al.* 1986; Kristiansen *et al.* 1997), a definitive set of US parameters are not yet defined. Therefore to determine US parameters for a stimulatory response in bone and dental tissues *in vitro*, it was necessary to evaluate cellular responses to a range of US frequencies and intensities. The present study therefore had two main aims:

1) Development of an *in vitro* US delivery method using a calibrated therapeutic US system, the DuoSon.
2) To compare the effects of different US frequencies and intensities on odontoblast-like and osteoblast-like cell cultures.

4.1 THERMAL EFFECTS FOLLOWING ULTRASOUND EXPOSURE USING THE DUOSON DEVICE

For clinical applications, a water coolant is used to minimise injury to pulpal and periodontal tissues when using dental ultrasonic scalers (Nicoll and Peters 1998) as absorption of US energy in tissues leads to heat production. This thermal effect is considered negligible during LIPUS application due to the acoustic wave being low in frequency, low in intensity and transmitted in a pulsed waveform (Khanna *et al.* 2009). To develop an *in vitro* US delivery method it was necessary to consider and evaluate the thermal influence of US. Three US frequencies and intensities available as settings on the DuoSon were therefore assessed for thermal impact on cell culture medium. The US frequencies, intensities and exposure times applied in this present study were similar to those previously applied for *in vitro* tissue repair including accelerated healing for osteoradionecrosis (Reher 1997; Reher *et al.* 1998) and for induction of neovascularisation *in vivo* (Ramli *et al.* 2009).

Temperature rise was initially evaluated in a non-temperature controlled set-up after exposure to US at low frequency and intensity settings available on the DuoSon. These US settings were 45kHz at an intensity of 25mW/cm^2, 1MHz at an intensity of 250mW/cm^2 and combined frequency (45kHz and 1MHz) at a combined intensity of 25mW/cm^2 and 250mW/cm^2. A temperature rise of 1°C, 2.5°C and 5.5°C was observed after a 5 minute exposure to 45kHz US at an intensity of 10mW/cm^2, 25mW/cm^2 and 75mW/cm^2 respectively (**Figure 4-1A**). Next, thermal effects of US were assessed using a temperature controlled approach. For this purpose, the

experiment was performed within an anti-reflective chamber that was maintained at 37°C using a plate heater (refer to **Section 2.1.2**). The results demonstrate that US at 1MHz produced the lowest overall temperature rise (1.5°C) following a 30 minute exposure using this set-up. 45kHz US delivery resulted in a temperature rise of 3.3°C after a 30 minute exposure, while combined frequency delivery generated the highest temperature rise of 4°C (**Figure 4-1B**).

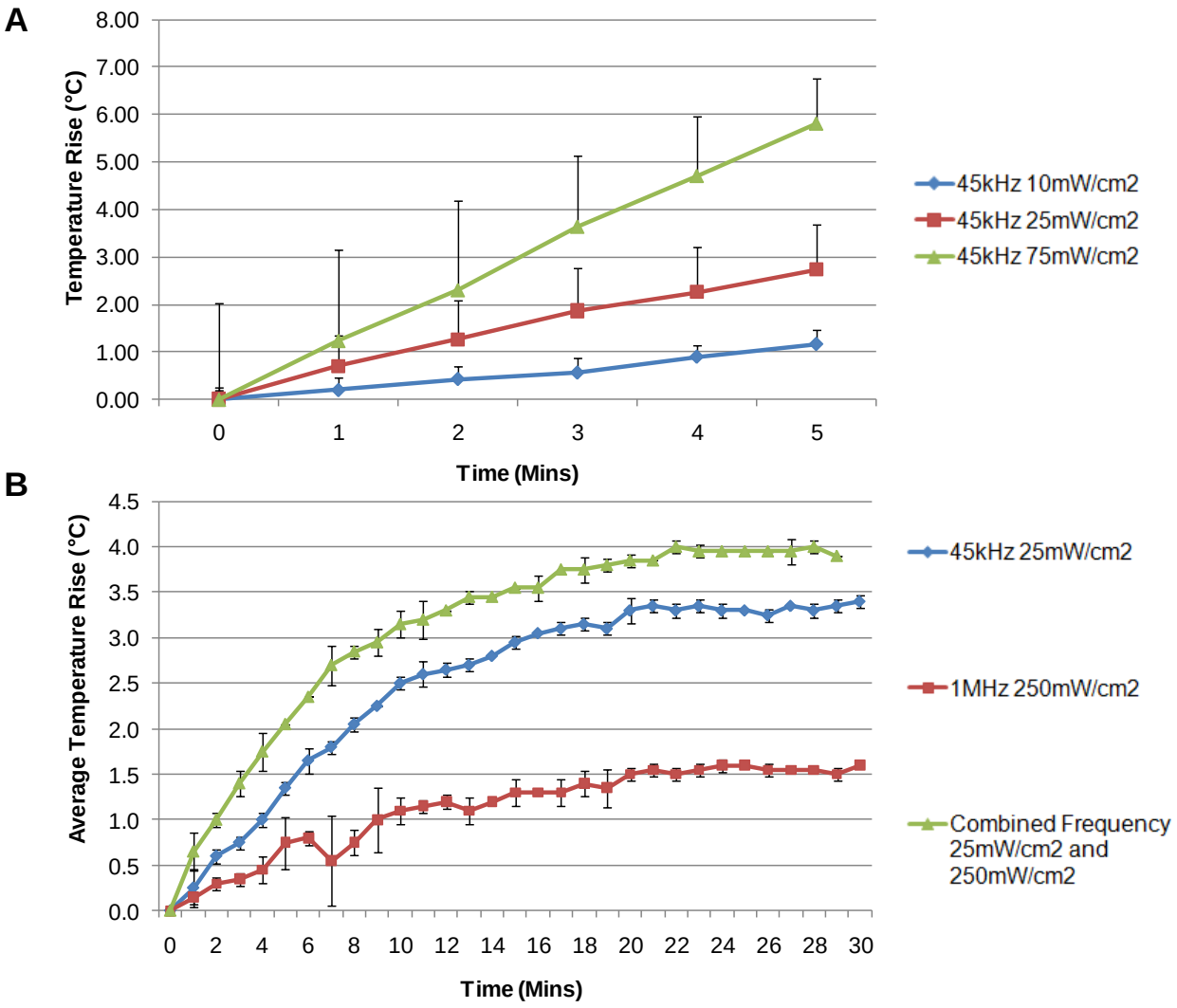


Figure 4-1 Temperature rise of cell culture medium during (A) 5 minutes of US application at 45kHz at three US intensities. Temperature rise was intensity dependent and resulted in a minimum temperature rise of 1°C at the lowest intensity available for the DuoSon. To create a stable experimental environment, an external temperature control of 37°C was incorporated into the experimental set-up. (B) After a 30 minute exposure, all frequencies and intensities applied resulted in less than a 4°C temperature rise in cell culture medium. Actual temperatures are plotted and data is expressed as mean of 3 experiments + SD.

4.2 INFLUENCE OF ULTRASOUND ON CELL VIABILITY

Cell viability was assessed using 0.4% trypan blue solution and demonstrated no significant effect of US on MDPC-23 cells after a single 15 or 30 minute US exposure, or following two 15 minute exposures of US at 45kHz, 1MHz and combined frequency (see **Figure 4-2A, B and C**). However, following two 30 minute US exposures, MDPC-23 cells exhibited a significant intensity dependent decrease in cell viability. A 54%, 75% and 79% loss of viable cells was observed in MDPC-23 cells exposed to US at 45kHz, 1MHz and combined frequency respectively when compared with control (see **Figure 4-2D**). MC3T3-E1 cell viability was not significantly affected after US exposure at all frequencies and exposure times applied.

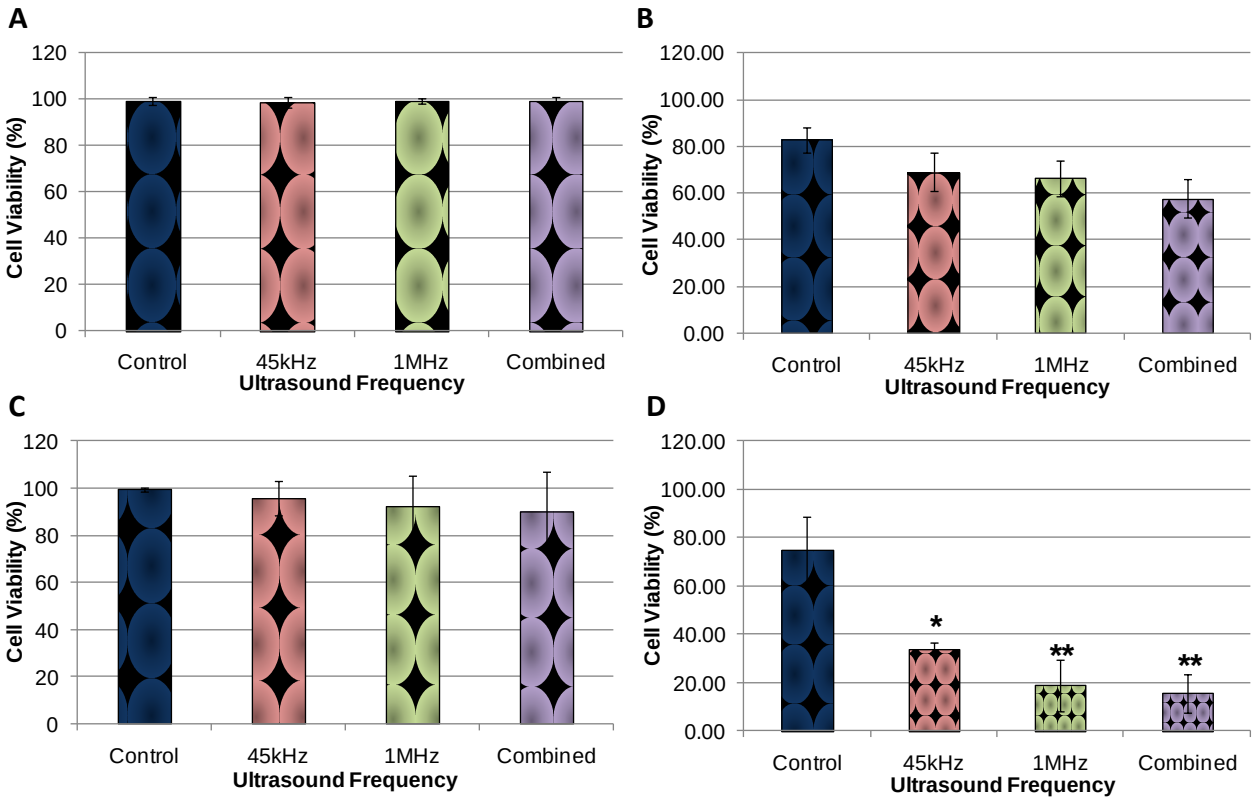


Figure 4-2 MDPC-23 cell viability as assessed by trypan blue exclusion staining assay. Ultrasound was delivered for (**A**) 15 minutes, **(B**) 30 minutes, (**C**) two 15 minute exposures and (**D**) two 30 minute exposures of US at 45kHz, 1MHz and combined frequency using the DuoSon device. A significant dose-dependent decrease in cell viability was evident in MDPC-23 cells stimulated with two 30 minute US exposures only. Data is expressed as mean percentage cell viability for 6 experiments ± SD. *P<0.05, **P<0.01 when compared with control group without US exposure.

4.3 EFFECTS OF ULTRASOUND EXPOSURE ON CELL PROLIFERATION

Exposure to LIPUS has been demonstrated to promote *in vitro* cell proliferation in a range of cell types including fibroblasts, osteoblasts and nucleus pulposus cells (Sena *et al.* 2005; Hiyama *et al.* 2007; Mostafa *et al.* 2009). Furthermore, US delivered at 45kHz has been shown to induce increased osteoblast proliferation *in vitro* comparable to that of osteoblasts stimulated with US at 1MHz (Reher *et al.* 1998). Cell proliferation as determined by cell count was therefore assessed after stimulation with three different US frequencies and intensities to determine the optimum frequency for maximal stimulation of cell number in MDPC-23 and MC3T3-E1 cells.

MDPC-23 cell cultures showed a significant increase in cell number following a single 30 minute exposure and two 15 minute exposures of US at 45kHz, 1MHz and combined frequency compared with control cultures without US stimulation. No significant increase in cell number was observed in MDPC-23 cells stimulated with a single 15 minute exposure or two 30 minute exposures (see **Figure 4-3A and D**). Ultrasound delivered at 1MHz resulted in the highest increase in cell number. In groups stimulated with 1MHz for a single 30 minute exposure (**Figure 4-3B**) and two 15 minute exposures (**Figure 4-3C**), increases in cell number of 40% and 30% respectively were observed. Similar increases in cell number were observed after US stimulation at 45 kHz and combined frequency. When MDPC-23 cells were stimulated for a single 30 minute US exposure using 45kHz or combined frequency

(**Figure 4-3B**), an increase in cell number of 30% and 38% were observed respectively. Two daily 15 minute US exposures (**Figure 4-3C**) at 45kHz and combined frequency resulted in a 20% increase in cell number 24 hours after US stimulation when compared with control.

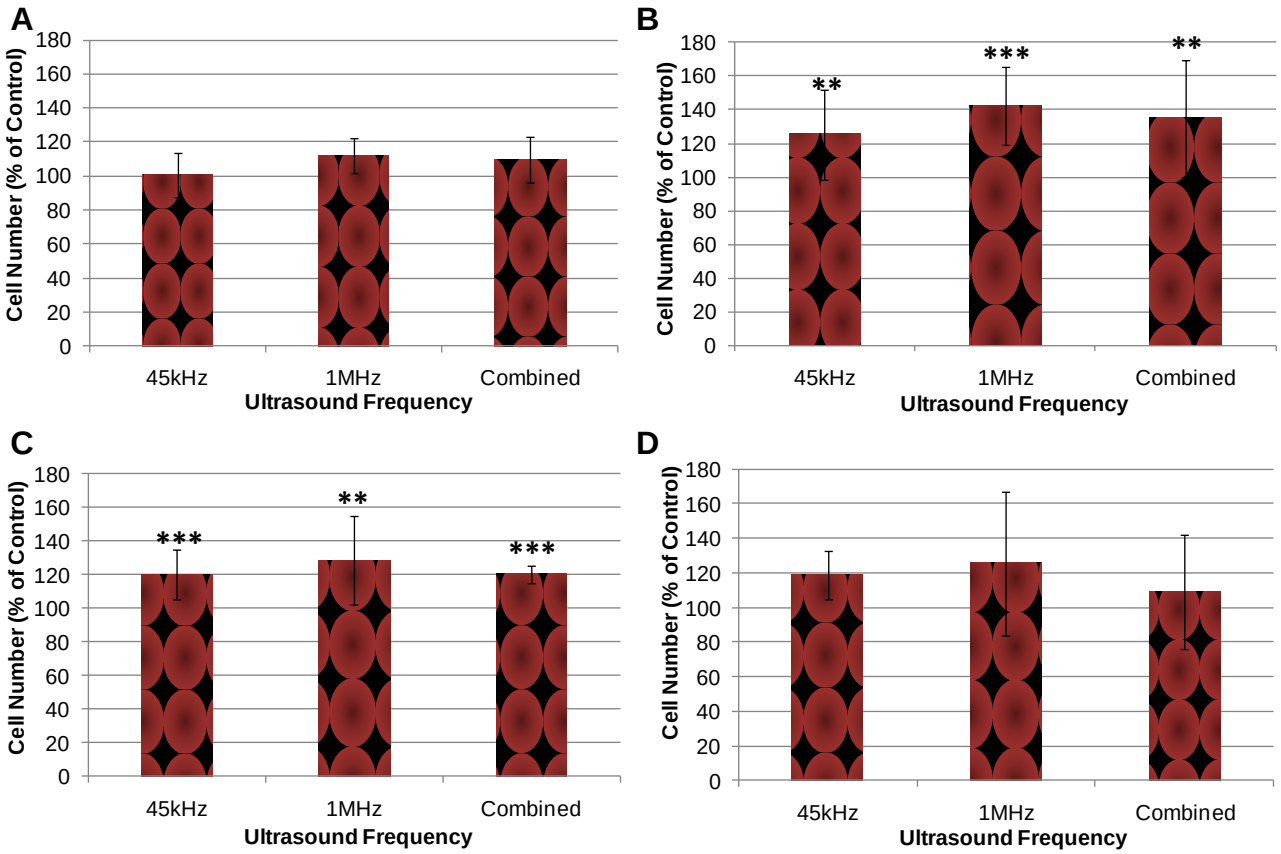


Figure 4-3: MDPC-23 cell counts performed 24 hours after (**A**) a single 15 minute exposure, (**B**) a single 30 minute exposure, (**C**) two 15 minute exposures and (**D**) two 30 minute exposures of US at a frequency of 45kHz, 1MHz and combined frequency. Increased cell number was observed under certain conditions and the most significant increase in cell population was observed in cells stimulated with 1MHz US when compared with control cells without receiving US. Data is presented as mean percentage of control for 5 experiments ± SD. **P<0.01, ***P<0.001 when compared with control group without US stimulation.

Enhanced cell proliferation in osteoblasts exposed to US at a frequency of 1.5MHz and an intensity of 30mW/cm^2 is well established (Sena *et al.* 2005; Takayama *et al.* 2007; Alvarenga *et al.* 2010). However, there are limited research studies that have evaluated the effect of kHz US stimulation in osteoblasts and it has been noted that kHz US may have several significant therapeutic advantages over MHz US stimulation including less heat production, shorter exposure times and treatment over a larger treatment area (Reher *et al.* 1998). In this present study, kHz US, MHz US and combined frequency were compared for their effect on MC3T3-E1 cell number. Only cells stimulated with US at 45kHz resulted in a significant increase in cell number at all doses applied. Furthermore, 45kHz US stimulated the highest increase in cell number when compared with cultures stimulated with 1MHz and combined frequency US. Cell number was increased by 34% after a single 15 minute exposure (**Figure 4-4A**), by 26% after a single 30 minute exposure (**Figure 4-4B**), by 22% after two 15 minute exposures (**Figure 4-4C**) and by 43% after two 30 minute exposures (**Figure 4-4D**) when compared with control. MC3T3-E1 cell number was significantly increased after stimulation with 1MHz US at certain doses (**Figure 4-4**) and US at combined frequency was not able to significantly enhance MC3T3-E1 cell number.

The application of US stimulation to MDPC-23 and MC3T3-E1 cell cultures significantly affected cell number under certain conditions with conditions being dependent on cell type as well as the frequency and intensity of the US wave. A significant intensity-dependent increase in cell number was observed in MC3T3-E1 cells when stimulated with US at 45kHz at all exposure times applied. This trend was

not observed in MDPC-23 cells where the most significant increase in cell number resulted after stimulation with 1MHz US at certain exposure times.

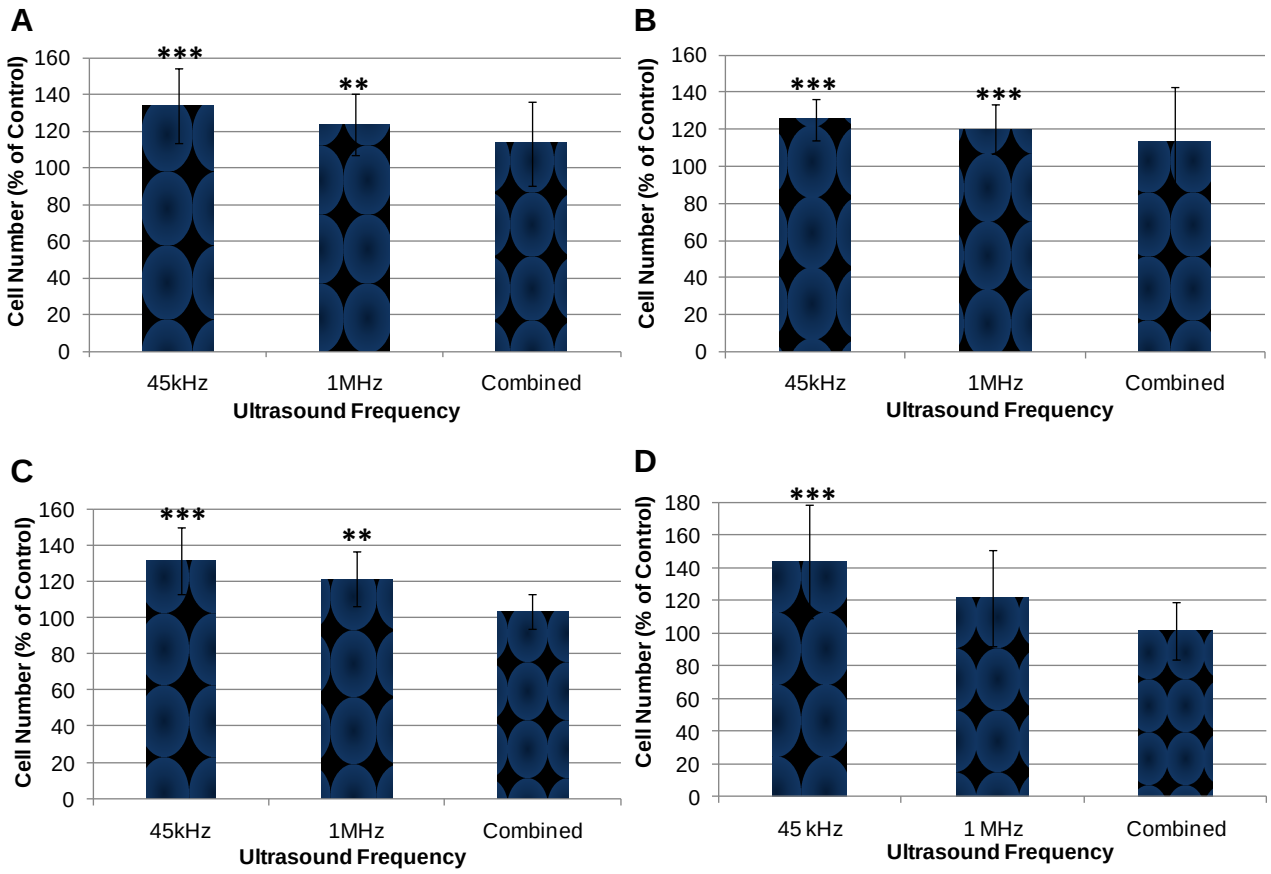


Figure 4-4: MC3T3-E1 cell count performed 24 hours after (**A**) a single 15 minute exposure, (**B**) a single 30 minute exposure, (**C**) two 15 minute exposures, and (**D**) two 30 minute exposures at 45kHz, 1MHz and combined frequency when compared with control cultures without US stimulation. A significant increase in MC3T3-E1 cell number was induced by 45kHz US which resulted in the highest increase in cell counts. Data is expressed as percentage of control for 6 experiments ±SD. **P<0.01, ***P<0.001 when compared with control group without US stimulation.

4.4 DISCUSSION

Therapeutic US is commonly applied using LIPUS which is in the lower MHz range to promote fracture healing (Kristiansen *et al.* 1997) and soft tissue repair (Enwemeka *et al.* 1990). Application of kHz US for tissue repair has been suggested to have advantages over conventional MHz US. In theory, kHz US is able to penetrate deeper tissue layers, reduce tissue heating, treat a larger surface area and require shorter exposure times (Williams 1983a). However, there is limited research evidence supporting the therapeutic value of kHz US and the existing evidence does not support the claims that kHz has a better effect than MHz US (Bradnock 1994; Reher 1997; Basso and Pike 1998). To evaluate the cellular responses in oral tissues exposed to dental ultrasonic scalers, a commercial US device was used to deliver US at three US frequencies comparable to those used for dental applications and for LIPUS applications. Additionally, combined frequency was evaluated, a unique and untested US parameter setting. The outcome of these experiments will provide a range of key parameters for peak proliferative response in odontoblast-like and osteoblast-like cells. Moreover, the data will further support the possibility of US being used as a regenerative tool for dental tissue repair.

Controlling experimental temperature is necessary to minimise heat-induced disruption of cellular processes including DNA synthesis (Warters and Roti Roti 1982), which may result in heat-activated cell death and apoptosis (Landry *et al.* 1986). Severe heat stress (above 42°C) may cause irreversible damage sufficient to sterilise the cell and when exposed to mild heat stress, cells are able to increase their

survival by overexpressing HSPs (Garrido *et al.* 2001). Ultrasound at all frequencies applied produced minimal influence on temperature after the maximum exposure time available on the DuoSon device using the present model for *in vitro* US delivery. Cell viability was adversely affected only after two 30 minute exposures on consecutive days and consequently most likely to be result of non-thermal, mechanical stress to the cells.

Several authors have reported increased cell proliferation following US exposure in conjunction with US-induced accelerated healing for osteoradionecrosis, cartilage tissue repair and orthodontically induced root resorption (Reher 1997; Dalla-Bona *et al.* 2008; Korstjens *et al.* 2008). However, there are conflicting reports that have stated an inhibitory effect in cell proliferation following US stimulation (Kaufman *et al.* 1977; Harle *et al.* 2001b). Exposure of MDPC-23 and MC3T3-E1 cells to US using the DuoSon device increased cell number over a short culture time. As cell viability was not significantly affected under the test conditions used, an increase in cell number indicated enhanced cell proliferation. Cell proliferation is an important phase in tissue repair and US has been shown to have a stimulatory effect on osteoblasts, fibroblast, and endothelial cells (Dyson and Brookes 1983; Mortimer and Dyson 1988; Ramirez *et al.* 1997). Increased expression of HSPs help limit the damage caused by stress and thus facilitates cell survival by upregulating cell proliferation (Arya *et al.* 2007). However, this present model for *in vitro* US delivery produced temperature increases below the threshold required for HSP expression and it is conceivable that enhanced MDPC-23 and MC3T3-E1 cell count after US stimulation was the result of increased proliferation modulated by a combination of non-thermal

effects of US including acoustic streaming and cavitation and not as a consequence of HSP activation.

The parameters used for therapeutic LIPUS were established from previous studies for fracture repair that has demonstrated for optimal fracture repair, US should be delivered as pulsed US at a frequency of 1.5MHz and an intensity of 30mW/cm^2 with a treatment duration of 20 minutes per day (Duarte 1983; Pilla *et al.* 1990; Wang *et al.* 1994). The most studied parameter for US is intensity (Li *et al.* 2002; Reher *et al.* 2002) whereas less is known about the importance of US frequency on cell response. This present study suggested that there was no significant difference between single and multiple US doses. Furthermore, MDPC-23 required a lower US frequency and intensity to enhance cell number when compared with MC3T3-E1 cells. This implies that the optimum US parameters differ for different cell lines and clearly more research is required to explore the cellular effects of altering US parameters. Fine-tuning of US parameters is important to attain the desired biological effects of US (Feril and Kondo 2004) and once optimum parameters have been established, US may be incorporated into clinical devices to facilitate dental tissue regeneration. Thus, it is envisaged that non-invasive, multifunctional US devices may be developed for dental diagnostics, repair and regeneration (Scheven *et al.* 2009b).

CHAPTER 5 RESULTS

CELL RESPONSES TO ULTRASOUND

LIPUS is increasingly used as a supplementary therapy to promote bone and wound healing (Rubin *et al.* 2001), however, the underlying mechanism for intracellular signal transduction is poorly understood. In the present study, low frequency, low intensity US was able to promote MDPC-23 and MC3T3-E1 cell populations with minimal change in temperature indicating that the mechanism was non-thermal in origin. Alterations in cell morphology have previously been identified in response to mechanical stimuli such as US (McCormick *et al.* 2006). Both osteoblasts (Meazzini *et al.* 1998) and dental tissue-derived cells (Dhopatkar *et al.* 2005) have been shown to alter their morphology and size when subjected to mechanical strain and stress. It has also been suggested that LIPUS may cause vibrations within the extracellular matrix thereby affecting the actin cytoskeleton (Roovers and Assoian 2003) and stimulating various receptors and adhesion factors on the cell surface (Zhou *et al.* 2004). The aim of the present study was to investigate the possible mechanisms for intracellular US signal transduction by using cell morphology as a marker for responsiveness in US-stimulated cells, examining the actin cytoskeleton in response to US stimulation as an indicator for integrin-mediated signal transduction and assessing US-induced β-catenin activity for Wnt activation as a possible candidate for intracellular US transduction.

5.1 MORPHOLOGICAL OBSERVATIONS

To determine whether intracellular US transduction is facilitated by changes in cell morphology, which may give rise to the cellular effects induced by US, MDPC-23 and MC3T3-E1 cells were assessed for changes to cell size and shape immediately after

US stimulation. Ultrasound exposure of MDPC-23 cells appeared to have altered the surface of plasma membranes by causing the edges to become blebbed and uneven, and alteration to the plasma membranes were dose-dependent when compared with control cells, which exhibited smooth and intact plasma membranes (**Figure 5-1**).

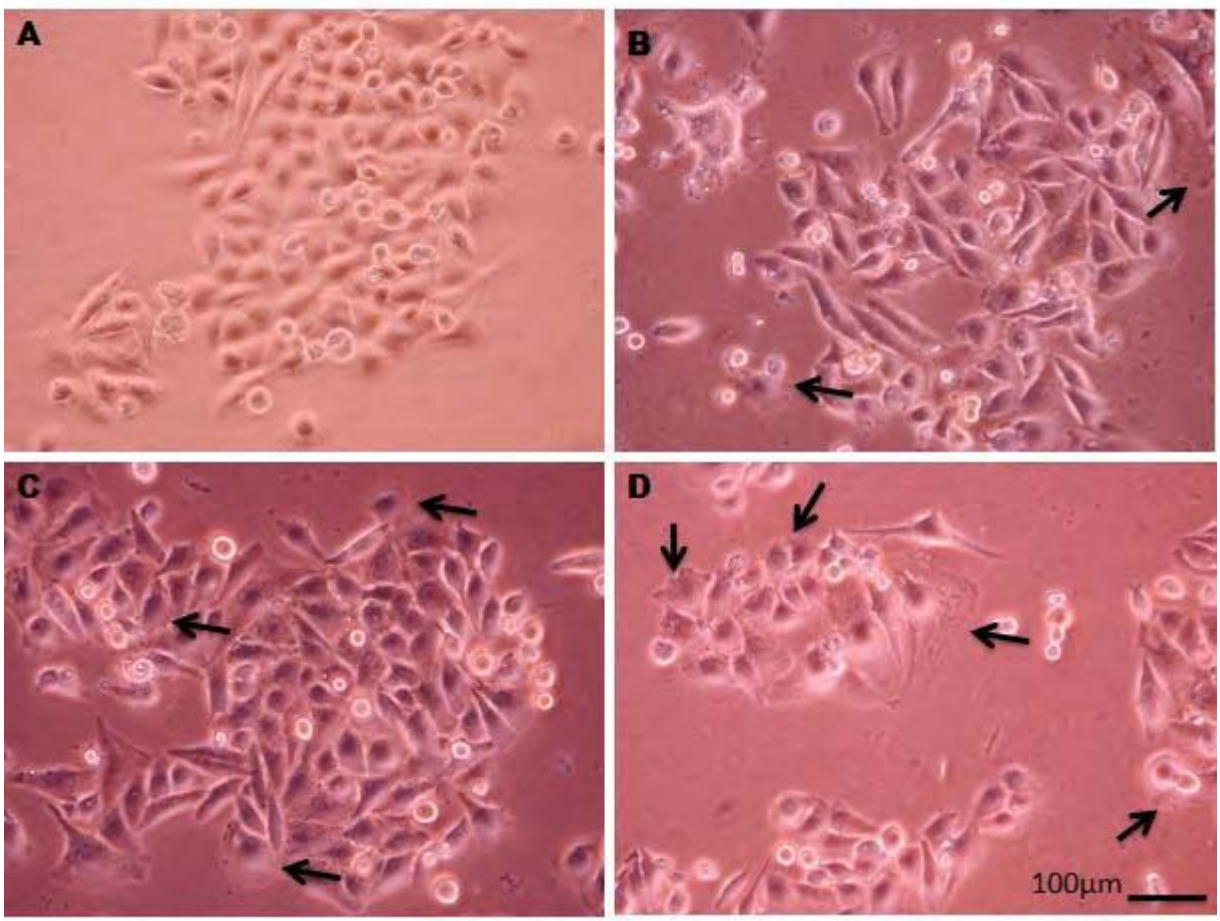


Figure 5-1 Phase contrast photomicrographs of MDPC-23 cell morphology after 30 minutes of US exposure. (**A**) Control cells displayed smooth cell surface whilst the number of cells displaying changes in surface changes of the plasma membrane (arrows) increased with increasing US frequency and intensity as (**B** = 45kHz, **C** = 1MHz and **D** = combined frequency respectively).

Approximately 30% of the MDPC-23 cells exposed to US at 45kHz resulted in surface alterations of the plasma membrane as assessed using Image J (see **Section 2.4**), whereas after US exposure at 1MHz, ~50% of US stimulated cells displayed uneven edges and blebbing of the plasma membrane surface. The majority of MDPC-23 cells displayed apparent cell surface alterations after US stimulation with combined frequency (**Figure 5-2A**). Ultrasound-stimulated MDPC-23 cells demonstrated a loss of uniformity in cell shape with increasing US frequency and intensity when compared with the cobblestone appearance visible in control cultures. Ultrasound-stimulated MDPC-23 cells also exhibited increased spreading and elongation with increasing US frequency and intensity when compared with control cells, which were uniform in size and shape (**Figure 5-2B**).

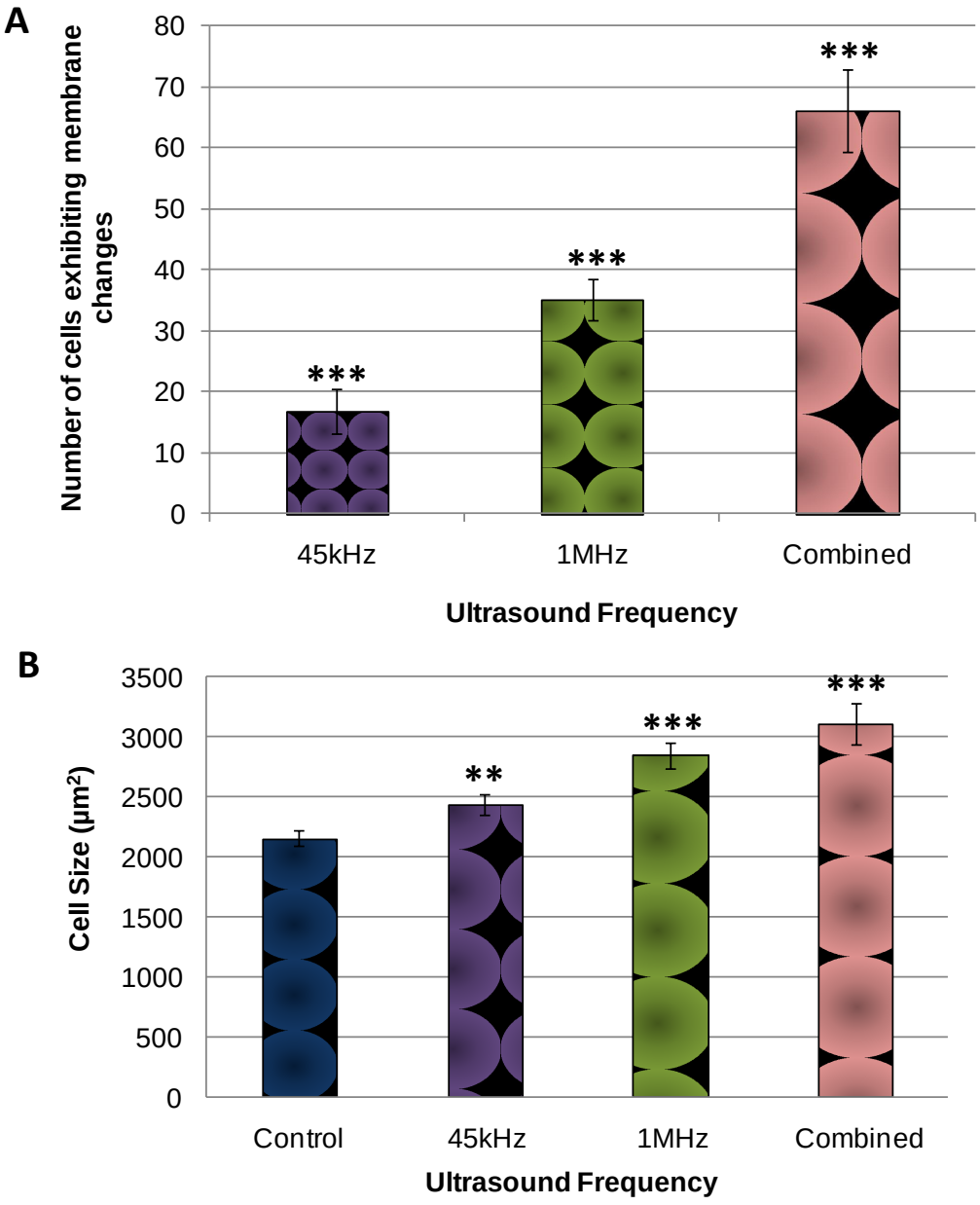


Figure 5-2 US-induced morphological changes were observed after 30 minutes of US exposure at 45kHz, 1MHz and combined frequency. Upon US stimulation, (**A**) a significant increase in the frequency of disrupted plasma membranes and (**B**) a significant intensity-dependent increase in cell size were observed in MDPC-23 cells as determined by ImageJ analysis. Data is represented as mean for 3 experiments ±SD.

In MC3T3-E1 cell cultures, exposure to US at 45kHz resulted in MC3T3-E1 cells exhibiting elongated processes with evidence of cell surface membrane disruptions along the processes (**Figure 5-3B**). When exposed to increasing intensities, MC3T3-E1 cell exhibited an increase in cell shape irregularity, most notably the appearance of thinner, more spindle-like processes extending from the cytoplasm, which appear to increase in frequency with increasing US intensity. Reduction in the size of the cell body was also evident in US-stimulated cells (**Figure 5-3B-D**). Furthermore, MC3T3-E1 cell shape appeared to become more polygonal with increasing US frequency and intensity when compared with control cells that exhibited a characteristic flattened shape. MC3T3-E1 cells in the control group were of equal size and length exhibiting distinct processes and cytoplasm that was clear from debris (**Figure 5-3A**).

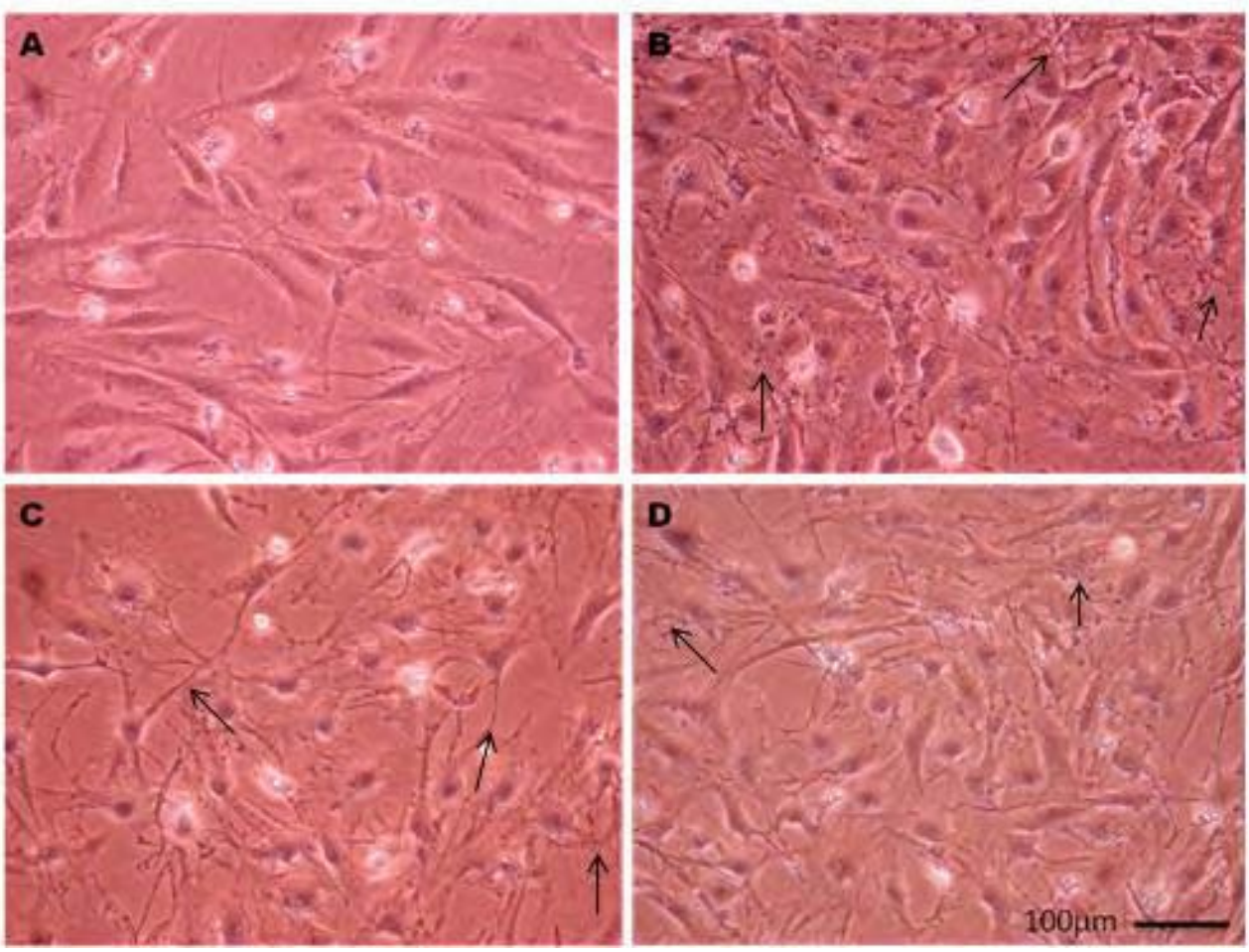


Figure 5-3 MC3T3-E1 cell morphology after US exposure at 45 kHz (**B**), 1 MHz (**C**) and combined frequency (**D**). Thinning of processes and the occurrence of disrupted cell surface membranes along these processes (arrows) increased with US frequency and intensity when compared with control cells (**A**).

5.2 ULTRASOUND EFFECTS ON ACTIN CYTOSKELETON

The ability for US to enhance cell proliferation rates for wound healing and fracture repair has been reported to be triggered by the activation of intracellular biochemical pathways induced by cytoskeletal alterations (Zhou et al. 2004; Hauser et al. 2008b). To determine the role of the cytoskeleton in intracellular US transduction, the organisation of cellular actin filaments were visualised after a 30 minute exposure of US at a frequency of 45kHz, 1MHz and combined frequency. No changes in actin polymerisation, as demonstrated by differences in fluorescent intensity, were evident in MDPC-23 and MC3T3-E1 cells after US exposure when compared with control. This was further verified by image analysis using ImageJ software (**Section 2.3.2.1**). A disruption to actin filament organisation was observed in US-stimulated MDPC-23 cells, which were classified by irregular patches of actin staining across the cytoplasm. In contrast, non-stimulated MDPC-23 cells exhibited an undisrupted, organised distribution of actin filaments. A notable difference between experimental and control groups in both MDPC-23 and MC3T3-E1 cells was the appearance of filopodia that extended from the cell surface within the US stimulated groups. In US-stimulated MDPC-23 cells, ruffle-like structures were observed at the cell periphery (**Figure 5-4**).

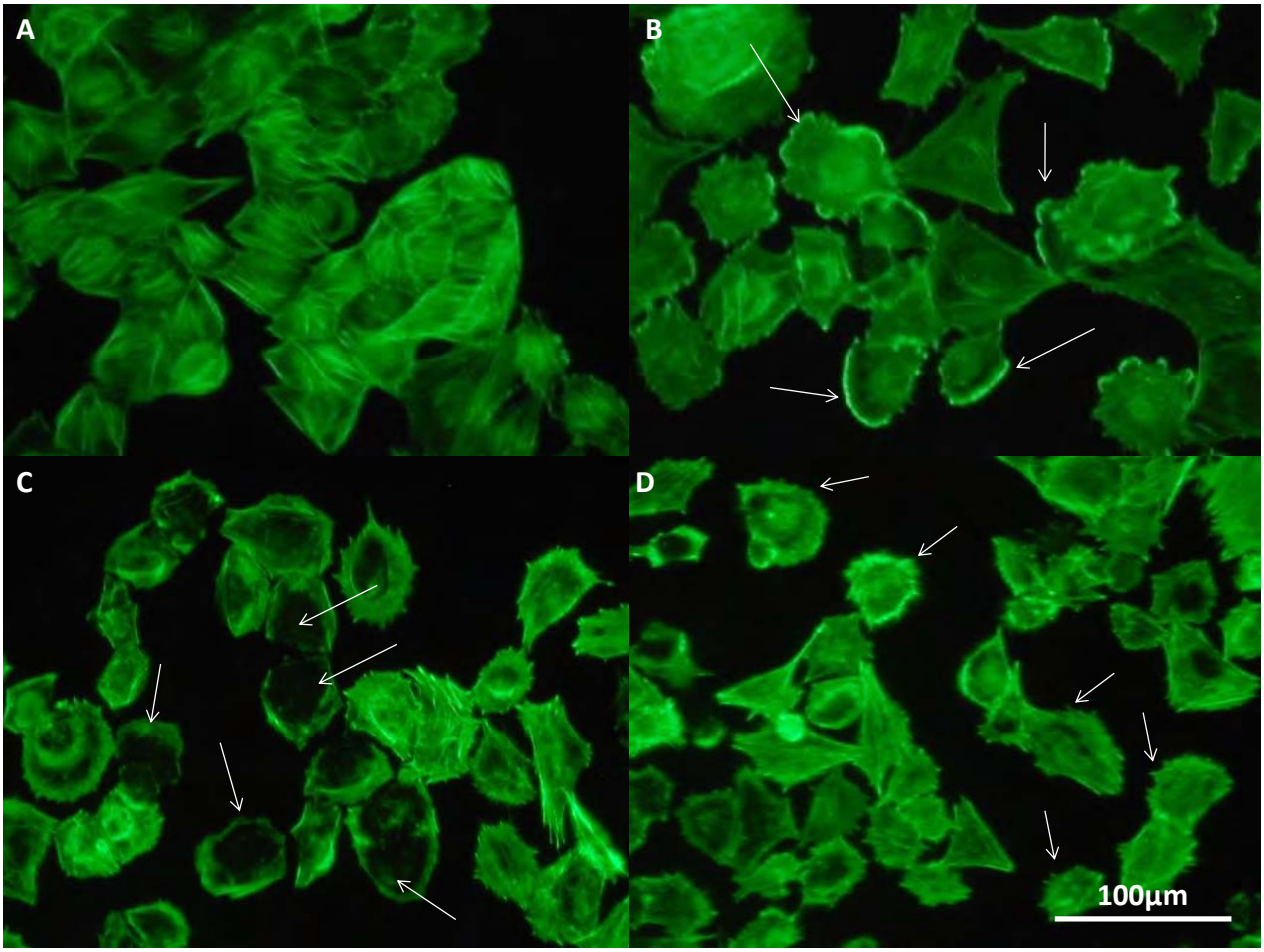


Figure 5-4: Fluorescent images of the actin cytoskeleton of MDPC-23 cells after a single 30 minute exposure of US. (**A**) Control cells exhibited smooth surface plasma membranes with undisrupted actin fibre organisation. Upon US stimulation, (**B**) ruffle-like structures at the cell periphery (arrows), (**C**) disruption of actin fibre organisation (arrows), and (**D**) the formation of filopodia extending from the cell surface were observed. Changes to the cell periphery and disruption to the actin cytoskeleton were dose-dependent. Images are representative of three experiments.

5.3 INTRACELLULAR ULTRASOUND SIGNAL TRANSDUCTION

The Wnt-signalling pathway has been implicated in LIPUS signal transduction in chondrocytes resulting in cell proliferation through activation of β-catenin (Takeuchi *et al.* 2008). The Wnt-signalling pathway may be associated with US-enhanced cell populations (**Chapter 4**) therefore changes to Wnt-signalling in MDPC-23 and MC3T3-E1 cells were also investigated by immunocytochemical analysis of β-catenin.

After a 30 minute exposure of US at 45kHz, 1MHz and combined frequency, MDPC-23 and MC3T3-E1 cells demonstrated positive cellular and nuclear β-catenin staining although the US-stimulated groups displayed the same degree of β-catenin staining compared with the control. However, differences in the intensity of β-catenin staining were evident between control and experimental groups in MDPC-23 cells 24 hours after US exposure. Cells exposed to 1MHz US exhibited the most intense staining of β-catenin when compared with cells exposed to 45kHz and combined frequency evident by the presence of more intensely stained nuclei and cytoplasm (**Figure 5-5A**). A modest difference in the intensity of β-catenin staining was also apparent in MDPC-23 cells 24 hours after exposure to US at 45kHz and combined frequency, however, 48 hours after US exposure, an increase in β-catenin was evident only in cells stimulated with 1MHz US. No observed difference in the intensity of β-catenin staining between control cells and cells stimulated with US at 45kHz and 1MHz 48 hours after exposure was detected (**Figure 5-5A**). There was minimal difference in

the intensity of β-catenin staining of MC3T3-E1 between the experimental and control groups at all timepoints studied (**Figure 5-5B**).

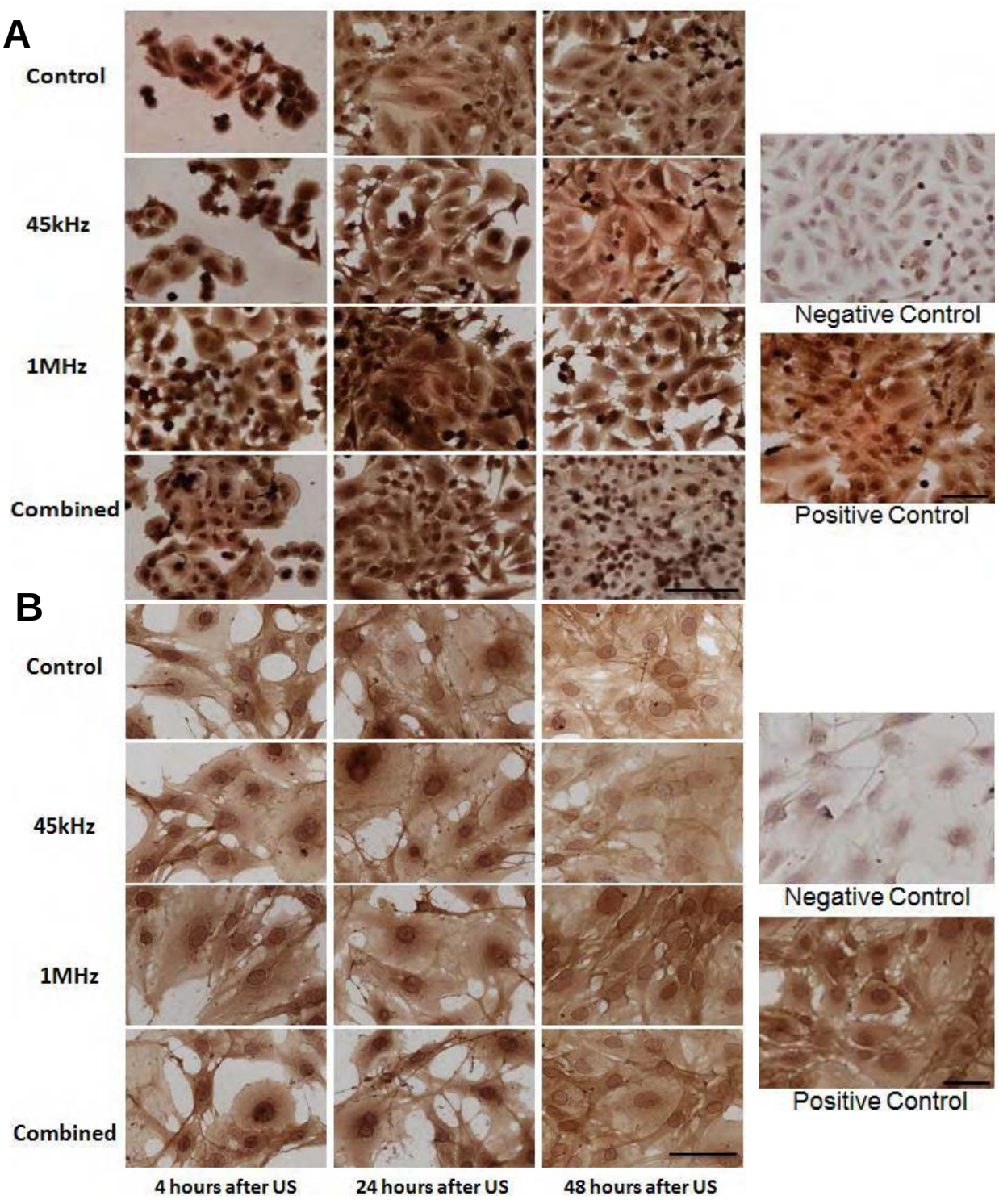

A
Control
45kHz
1MHz
Combined
Negative Control
Positive Control
B
Control
45kHz
1MHz
Combined
Negative Control
Positive Control
4 hours after US
24 hours after US
48 hours after US

Figure 5-5: Immunocytochemical analysis of β-catenin staining in (**A**) MDPC-23 cells and (**B**) MC3T3-E1 cells 4, 24 and 48 hours after US stimulation. Light microscopy images indicated no difference in staining between all timepoints when compared to control at all frequencies applied. Scale bar represents 100μm.

5.3.1 DISCUSSION

The cellular mechanism by which US elicit favourable biological responses on the repair of damaged tissue remains to be established. *In vitro* studies have suggested that integrins are responsible for the conversion of extracellular US stimuli to intracellular biochemical signals thereby regulating cell responses including viability and proliferation (Aplin *et al.* 1998; Giancotti and Ruoslahti 1999; Zhou *et al.* 2004; Tang *et al.* 2006). Signal transduction may also occur through structural alterations of the intracellular cytoskeleton and there is growing evidence that the changes within the cyto-architecture of the cell and the activation of signal transduction pathways are linked (Baskin *et al.* 1993; Ingber *et al.* 1994; Toma *et al.* 1997). Periodontal ligament fibroblast-like cells and osteoblasts have been shown to alter their morphology and size when subjected to mechanical strains and stresses (Roberts *et al.* 1981; Meazzini *et al.* 1998; Hatai *et al.* 2001; Chiba and Mitani 2004). Indeed, elongated cell shape and changes in the alignment of actin fibres was observed in osteoblasts after LIPUS, which can be regarded as a form of non-physiological mechanical stimulation (Meazzini *et al.* 1998; Zhou *et al.* 2004; McCormick *et al.* 2006); however, there are no studies to date on the effect of US on odontoblast-like cell morphology and intracellular US transduction.

In this study, distinct changes in cell morphology and the actin cytoskeleton were observed after US exposure in MDPC-23 and MC3T3-E1. The alterations in cell surface membrane structure and disruption to the actin cytoskeleton may indicate signs of cell injury (Cobb *et al.* 1996; White *et al.* 2001). Changes in cell membrane

architecture after US stimulation *in vitro* has previously been noted and researchers have postulated that the non-thermal effects of US may influence cell function by reversibly affecting the permeability of the plasma membrane (Dyson 1982; ter Haar 1999) which then initiates a cellular recovery response characterised by increased cell proliferation and protein production (Maeda and Murao 1977; Webster *et al.* 1980). Indeed, in the present study, cells stimulated with a single 30 minute exposure to US did not demonstrate irreversible damage and were accompanied by significant changes in cell number (**Chapter 4**). It is conceivable that the alterations in cell surface membranes may play a pivotal role in US-enhanced cell numbers of MDPC-23 and MC3T3-E1 cells.

The precise mechanism by which US promotes the proliferation of cultured MDPC-23 and MC3T3-E1 cells described in **Chapter 4** remains to be clearly elucidated however, US signal transduction to the nucleus has been suggested to occur via integrin-associated pathways, in particular the phosphatidylinositol 3-OH kinase/Akt (PI3K/Akt) pathway (Tang *et al.* 2006; Takeuchi *et al.* 2008). PI3K subsequently regulates β-catenin activity, which also plays a pivotal role in the Wnt signalling pathway and is an important regulator of bone and tooth development (Akita *et al.* 1996; Thesleff and Sharpe 1997). Furthermore, Wnt signalling has been reported to be activated upon LIPUS stimulation via the PI3K/Akt pathway in human MG-63 osteoblastic cells (Olkku *et al.* 2010). In this present study, US-stimulation of MDPC-23 cells resulted in elevated β-catenin staining. Interestingly, elevation of β-catenin activity and an increase in cell proliferation was also observed in LIPUS-treated chondrocytes (Takeuchi *et al.* 2008). Increased MDPC-23 cell number, as described

in **Chapter 4**, may be regulated by the Wnt/β-catenin pathway. To further investigate this possibility, it is necessary to examine β-catenin specific transcriptional targets such as nuclear factor kappa B which coordinates the expression of genes that control cell proliferation (Deng *et al.* 2004). Enhanced β-catenin staining by US stimulation in MDPC-23 cells was transiently expressed for 24 hours and returned to background levels 48 hours after US exposure. To maintain enhanced β-catenin expression, stimulation of MDPC-23 cells with 1MHz US every 24 hours may be required.

Activation of the Wnt/β-catenin pathway has been suggested to enhance mineralised tissue regeneration (Scheller *et al.* 2008). Furthermore, LIPUS has previously been shown to stimulate β-catenin translocation in chondrocytes (Takeuchi *et al.* 2008). However, in this present study, β-catenin staining was not evident in US-stimulated osteoblast-like cultures. Subtle changes in staining within experimental groups may have been overlooked due to the high background staining observed in control cells. Photomicrographs of MC3T3-E1 cells suggested a higher β-catenin activity in the unstimulated control group than in the Wnt3a-induced positive controls. It is conceivable that in this present study, the Wnt/β-catenin pathway may have been induced by an additional factor as well as by US stimulation. The Wnt/β-catenin pathway has previously been shown to be enhanced by vibrations in chondrocytes (Takeuchi *et al.* 2006). The vibrations of the laminar flow hood in which the experiment was executed may have provided the stimulus needed to exaggerate β-catenin staining in MC3T3-E1 cells for both control and experiment groups. To isolate

US-mediated pathways, the use of anti-vibration tables may be required for future studies.

In conclusion, the pathway which governs intracellular US transduction remains inconclusive however; this present study demonstrates that US may exert its effects on cellular functional activities by stimulating the Wnt-signalling pathway to induce alterations in cell morphology and the actin cytoskeleton. The activation of the Wnt-signalling pathway by US may provide supporting evidence for US being used as a therapeutic tool in dentistry as Wnt-signalling has a key role in tooth development and therefore conceivably in dental tissue repair (Sarkar and Sharpe 1999). Furthermore, Wnt signalling has been found to be colocalised with DSPP expression in odontoblasts and therefore may be linked to regulating dentinogenesis (Yamashiro *et al.* 2007). Ultrasound-activated Wnt signalling could therefore provide an opportunity for exploitation to stimulate dentinogenesis for dental tissue repair.

CHAPTER 6 RESULTS

INFLUENCE OF ULTRASOUND ON IN VITRO SCRATCH WOUND HEALING

LIPUS has been used as a therapeutic tool to accelerate the healing of soft and hard tissue wounds (Uhlemann *et al.* 2003; Breuing *et al.* 2005; Stanisic *et al.* 2005). In dentistry, LIPUS has been shown to accelerate periodontal wound healing *in vivo* (Ikai *et al.* 2008) and the use of LIPUS as a safe and effective method for oral ailments including reducing anti-inflammatory effects post-oral surgery and in the management of osteoradionecrosis, has been supported by other areas of *in vivo* research (Efanov 1981; ElHag *et al.* 1985; Harris 1992; El-Bialy *et al.* 2004). At a cellular level, LIPUS can stimulate increased growth factor expression (Kobayashi *et al.* 2009; Suzuki *et al.* 2009a), activate immune cells to migrate to the site of injury (Tezel *et al.* 2005) and enhance cell proliferation in human pulpous c ell lines (Kobayashi *et al.* 2009), cementoblasts (Kobayashi *et al.* 2009) and in osteoblast-like cells (Doan *et al.* 1999; Li *et al.* 2002; Hayton *et al.* 2005), all of which are processes key to tissue repair.

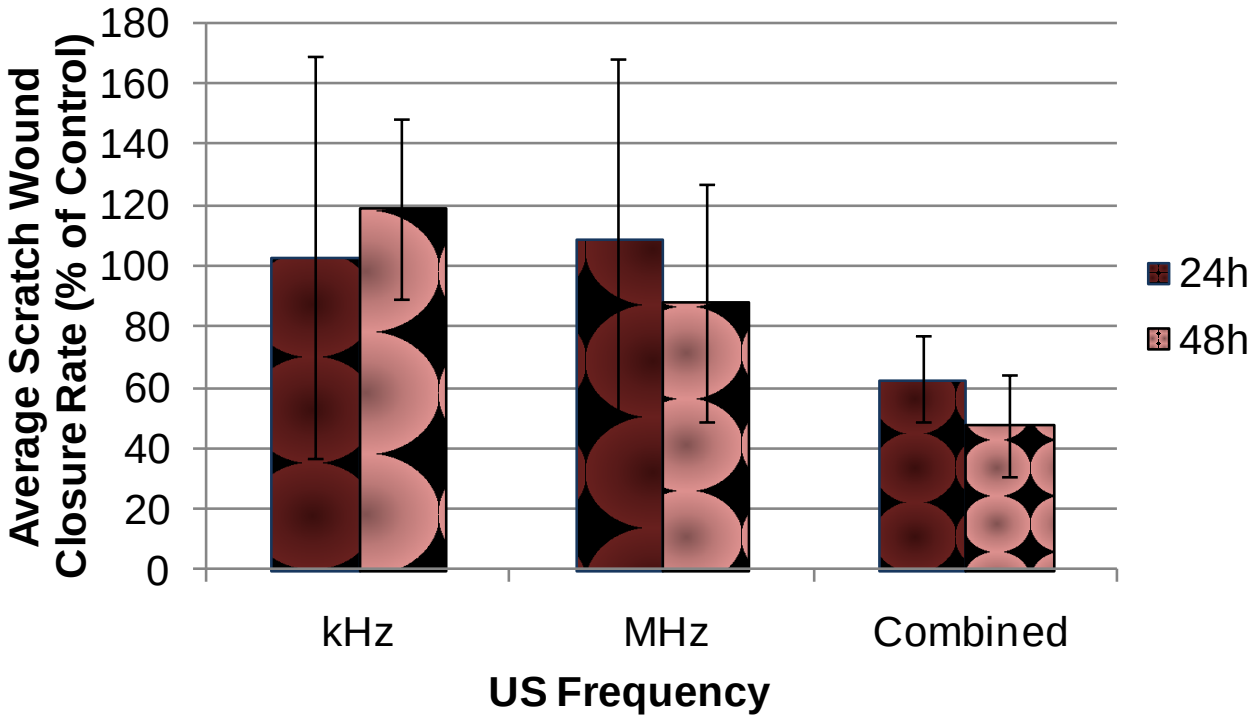
During reparative dentinogenesis in the pulp, dental pulp stem/progenitor cells migrate to the injured site and proliferate and differentiate into endothelial cells for angiogenesis (Mathieu *et al.* 2005), or into odontoblast-like cells for reparative dentine formation (Tecles *et al.* 2005). In this study, US-treatment of odontoblast-like cells up-regulated expression of the angiogenic growth factor VEGF (**Chapter 3**) and increased cell numbers (**Chapter 4**) suggesting potential for inducing repair processes. To further examine the role of US in pulp wound healing, the present study used the scratch wound assay for quantitative determination of US effects on MDPC-23 cell proliferation and migration, processes necessary for dentine regeneration (Mitsiadis and Rahiotis 2004). Accelerated bone healing by LIPUS

stimulation is well characterised and *in vitro* studies have suggested that these LIPUS effects may be mediated by promoting osteoblast proliferation (Reher *et al.* 1998), migration (Iwai *et al.* 2007), matrix synthesis(Reher 1997) and mineralisation (Harle *et al.* 2001a). In order to better understand the effects of US on osteoblast-mediated fracture repair, this present study also analysed, the effects of US on scratch wound healing in osteoblast-like cell cultures.

Mechanically scratching confluent cell monolayers results in movement and proliferation of cells into the denuded area (Sholley *et al.* 1977; Gotlieb and Spector 1981). The scratch wound assay is therefore considered a convenient and inexpensive method to study migration and proliferation in an artificial wounded area. In this study, MDPC-23 and MC3T3-E1 cell cultures were stimulated with US and then mechanically scratched using a p200 pipette tip **(Section 2.5)** to create an artificial wound. The wound healing rate was analysed by tracing the area of the denuded area using ImageJ software of images at daily intervals post-wounding (**Section 2.5.1**).

US treatment of MDPC-23 cells did not affect wound closure rate (**Figure 6-1A**), however scratch wound closure rates were significantly enhanced in MC3T3-E1 cells following a 30 minute exposure to US at all frequencies applied when compared with control wounds (**Figure 6-3B**). Enhanced wound closure rate was frequency dependent 24-hour after initial stimulation. Combined frequency US resulted in the greatest increase in wound closure rate that was five times greater than control.

Wound closure rate increased by 2.4-fold and 1.8-fold in scratch wounds stimulated with MHz and kHz US respectively.


Average Scratch Wound Closure Rate (% of Control)
180
160
140
120
100
80
60
40
20
0
kHz
MHz
Combined
US Frequency
24h
48h

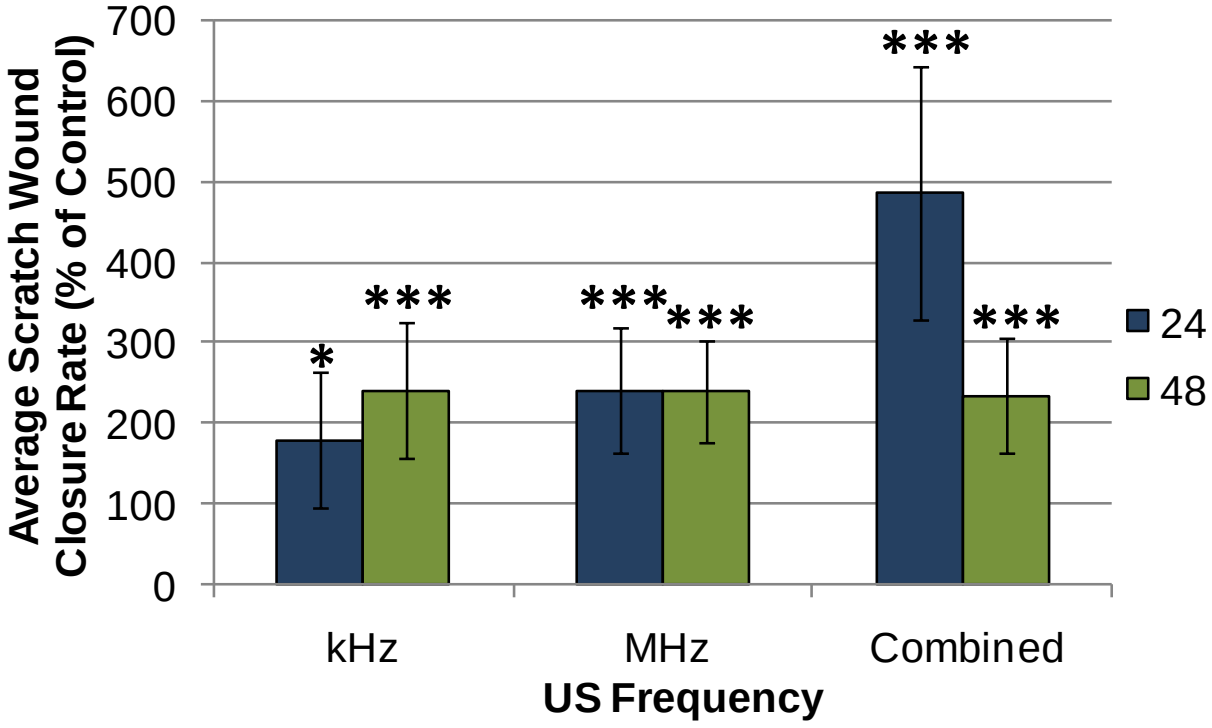

Average Scratch Wound Closure Rate (% of Control)
700
600
500
400
300
200
100
0
*
***
***
***
***
***
kHz
MHz
Combined
US Frequency
24
48

Figure 6-1 *In vitro* scratch wound closure rate 24 and 48 hours following a 30 minute US exposure at 45kHz, 1MHz and combined frequency. (**A**) US treatment of MDPC-23 demonstrated no significant effect on scratch wound closure rate however, (**B**) a significant increase was demonstrated in US-stimulated MC3T3-E1 scratch wounds when compared with sham-treated controls. A frequency dependent increase in wound closure rate was observed 24 hours after initial US stimulation where combined frequency US resulted in the stimulation of the highest scratch wound closure rate. Data is presented as mean rate from triplicate measurements for 3 experiments ± SD. *$P<0.05$, *$P<0.001$ when compared with sham-treated scratch wounds.

In vitro wound closure rate is influenced by both proliferative and migratory activities (Coomber and Gotlieb 1990) . To investigate the relative contributions of proliferation and cell migration to enhanced scratch wound closure in MC3T3-E1 cells, analyses were performed in the presence of mitomycin C, an inhibitor of cell proliferation (Wu *et al.* 1999; Ponchio *et al.* 2000). To establish the most appropriate dose of mitomycin C applicable for the suppression of cell proliferation whilst maintaining cell viability, the dose response to mitomycin C was evaluated using WST-1 assay in MC3T3-E1 cells every 24 hours for a total of 5 days after initial application. Data indicated that proliferation and viability was considerably decreased at applied mitomycin C concentrations above 0.05µg/ml. At 0.05µg/ml of mitomycin C, MC3T3-E1 cell number was comparable with the initial seeding density for the duration of the experiment and cell viability was maintained (**Figure 6-2**). Pre-incubation of MC3T3-E1 scratch wounds with mitomycin C at a concentration of 0.05µg/ml was therefore used to inhibit cell proliferation to assess the contribution of cell migration in US-enhanced *in vitro* scratch wound closure.

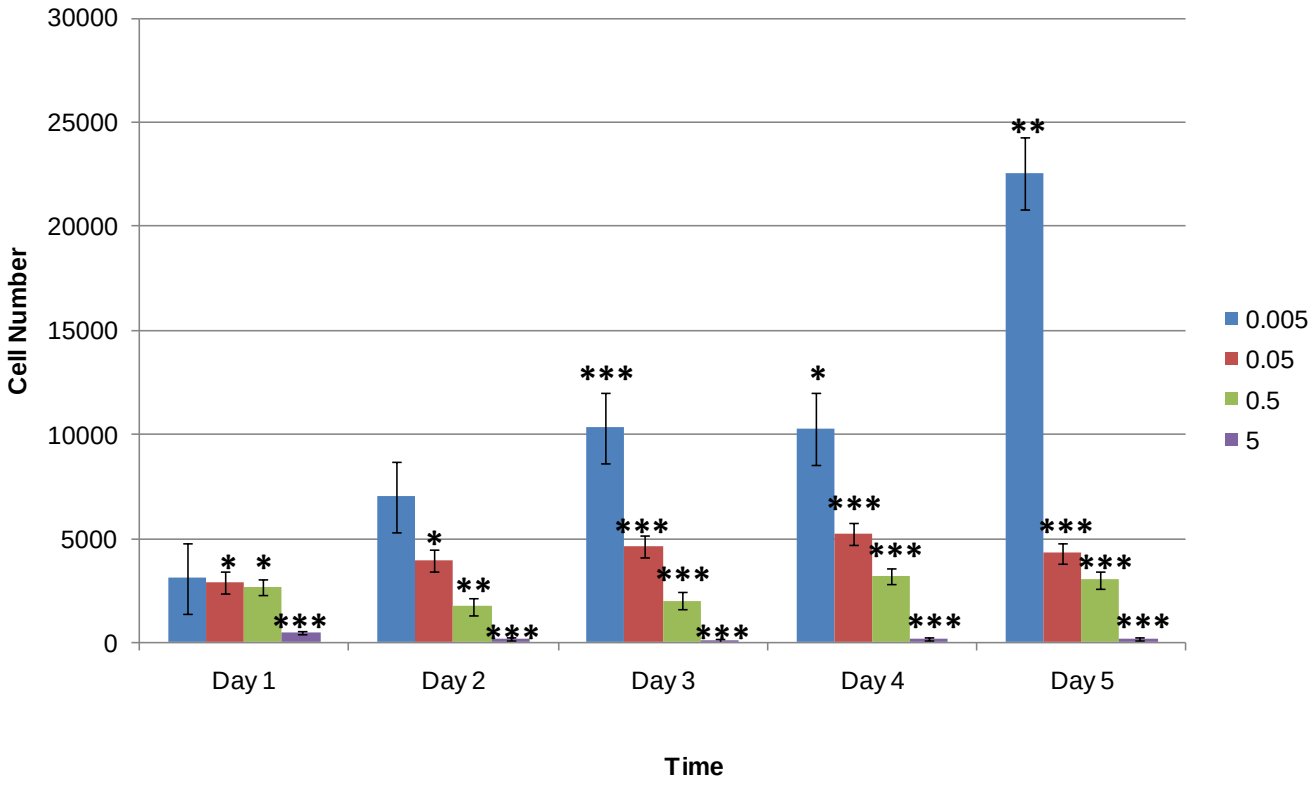


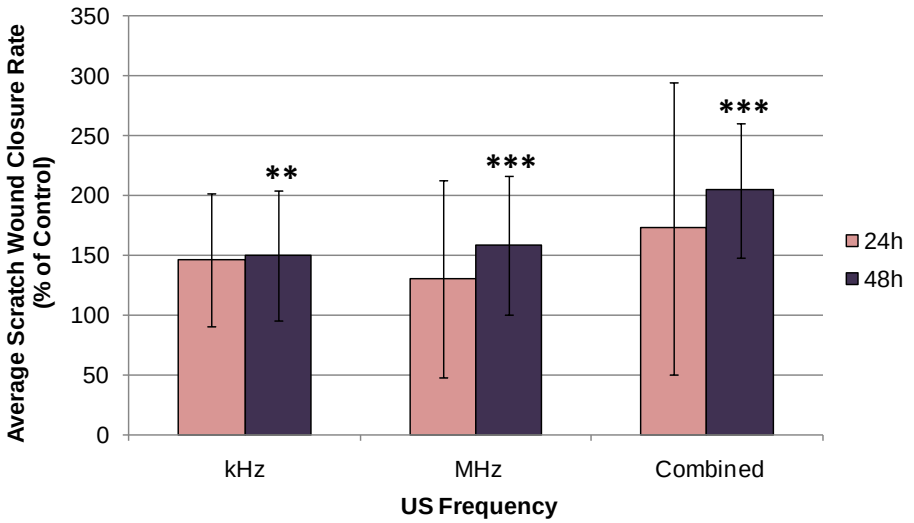
Figure 6-2: Effect of MC3T3-E1 growth in the presence of increasing concentrations of mitomycin C (µg/ml). Cell number remained at initial seeding density levels for 5 days without loss of viability in cultures with 0.05µg/ml of mitomycin C as verified by WST-1 assay. Data is presented for 4 experiments as mean viable cell number ±SD. *P<0.05, **P<0.01 and ***P<0.001 respectively relative to control.

Despite inhibition of proliferation, US was able to significantly stimulate an increase in scratch wound closure rate 48 hours after initial US stimulation at all frequencies applied (**Figure 6-3A**). Once again, a frequency-dependent increase in wound closure rate was observed in MC3T3-E1 scratch wounds 48 hours after initial US stimulation as previously seen for parallel experiments without mitomycin C. However, in contrast to the results in the absence of mitomycin C, no statistically

significant changes in scratch wound rate were detected 24 hours after initial US exposure. This discrepancy is potentially explained by variation in the scratch wound closure rate caused by differences in scratch wound width, which inhibits consistent results hence the large error bars (Pinco *et al.* 2002; Yue *et al.* 2010). As such these irregular starting conditions complicate the data analysis and make it difficult to directly compare experimental conditions.

When MC3T3-E1 scratch wound closure rate in the presence of mitomycin C was compared with those cultured in the absence of mitomycin C, scratch wound healing rate was significantly decreased by 20% in MC3T3-E1 scratch wounds 48 hours after exposure to 1MHz US (**Figure 6-3B**). This suggested that the enhanced wound healing effect of US in MC3T3-E1 cells exposed to MHz US may have been predominantly due to the influence of cell proliferation rather than migration. No significant change in scratch wound rate was detected in wounds treated with kHz and combined frequency US, which suggested that US-accelerated scratch wound closure at these frequencies were mainly due the influence of US-enhanced migration rather than proliferation.

A



B

		Average Scratch Wound Closure Rate (µm/h)	
		MC3T3	
US Frequency	**Timepoint (h)**	**Without Mitomycin C**	**With Mitomycin C**
Control	0	0.00	0.00
	24	7.72	8.93
	48	6.58	5.93
45kHz	0	0.00	0.00
	24	8.82	11.46
	48	9.85	9.10
1MHz	0	0.00	0.00
	24	13.36	11.27
	48	11.70	9.37*
Combined	0	0.00	0.00
	24	16.76	10.52
	48	10.28	8.46

Figure 6-3 Effect of US on the migratory of MC3T3-E1 scratch wound closure rates in the presence of mitomycin C. (**A**) A significant dose-dependent US-enhanced migration was demonstrated in MC3T3-E1 scratch wounds 48 hours after initial US exposure where exposure to combined frequency US resulted in the highest wound closure rate. (**B**) A significant decrease in scratch wound healing rate was observed in MC3T3-E1 scratch wounds cultured in the presence of mitomycin C when exposed to MHZ US, which suggested that proliferation may have contributed to US-enhanced wound closure. Data is presented as mean for 3 experiments ±SD. *P<0.05, *P<0.001 when compared with control.

6.1 DISCUSSION

Currently, LIPUS is being investigated as a non-invasive therapeutic approach for accelerated periodontal wound healing following flap surgery (Ikai *et al.* 2008), accelerated bone formation in osteodistraction sites (El-Bialy *et al.* 2003b; El-Bialy *et al.* 2008) and enhanced healing of orthodontically induced tooth resorption (El-Bialy *et al.* 2004). LIPUS may therefore be able to promote repair processes within the dentine-pulp complex and potentially facilitate the regeneration of dentine by providing the signals necessary to initiate reactionary dentinogenesis or recruit newly differentiated odontoblast-like cells for reparative dentine formation (Scheven *et al*, 2009). The advantages of promoting dental tissue repair are significant since they not only maintain or improve pulp vitality and therefore tooth viability, but also aid restoration of the normal tissue architecture of the tooth thereby relinquishing the need for expensive restorative dental procedures.

In this present study, US significantly enhanced *in vitro* scratch wound repair in an osteoblast model by a combination of enhanced cell migration and cell proliferation. Both effects are prerequisites for bone healing and in this present study; their role in wound repair is dependent on US frequency and intensity. Here, MHz US enhanced MC3T3-E1 wound repair by enhancing cell migration and is in agreement with other reports that has demonstrated accelerated repair of MC3T3-E1 wounds (Iwai *et al.* 2007) and repair of other tissue types (Mizrahi *et al.* 2007) were due to enhanced migration. Arguably, the US intensity, duration of treatment and exposure system differed between these studies, which may have contributed to the differences in

results. Interestingly, MC3T3-E1 cells exposed to kHz US at the same treatment time of 30 minutes resulted in the highest proliferative effect as described in **Chapter 4**, which suggests that US may enhance tissue repair by modifying both proliferative and migratory activities. This highlights the multifactorial beneficial effects of US yet also implicates the reasons why resolving the precise mechanism by which US influences cell behaviour is difficult and multiple biological mechanisms are most likely.

Exposure to US failed to increase the rate of MDPC-23 wound repair in this present study however, US may induce dentine repair in odontoblast-like cells. Although the scratch wound assay did not show US to accelerate wound closure, previous chapters have shown US to have significant influence on cell number and induction of angiogenic responses in odontoblast-like cells, which is suggestive of repair responses. Furthermore, the *in vitro* scratch wound assay may not be the best model for assessing the reparative capabilities of US-stimulated odontoblast-like cells as a cascade of biological events are initiated during dentine-pulp repair and further maintained by precise coordinated events within the surrounding dentine matrix or by pulpal responses (Smith 2003). Consequently, an *ex vivo* tooth organ slice model may be a better approach to determine the US-induced responses to injured dentally-derived cells (Sloan *et al.* 1998). Complimentary to the scratch wound assay, a molecular approach to assess the chemotactic agents released from the wounded cells, which trigger the repair mechanisms may also be studied. This present study however confirms the potential beneficial effects of US to accelerate tissue healing in bone.

In dentistry, US may have significant benefits in maxillofacial bone healing as evidence is already available to support the value of US for osseointegration following endosseous implants (Tanzer *et al.* 2001), and as a possible treatment for osteoradionecrosis by enhancing bone growth and through improving and promoting vascularisation (Reher 1997; Yan *et al.* 2011). This present study is the first to isolate the effects of US on cell migration from those of cell proliferation in odontoblast-like and osteoblast-like cells underscoring a possible mechanism by which US influences tissue repair. Furthermore, the enhancement of wound repair in MC3T3-E1 cells can be maximised by optimising US parameters such as frequency and exposure however, changing such parameters can also influence the mechanisms that control cell behaviour and is potentially the explanation for the variation of US effects for the same cell or tissue type cited within the literature. Indeed, a better understanding is needed of the relationship between US parameters and cell behaviour, which is key to elucidating the mechanisms behind the beneficial effects of US and to devise optimal strategies for US-based therapy. Only then can the role of US be fully supported as a therapeutic tool for dental tissue repair.

CHAPTER 7 RESULTS

EFFECT OF LOW FREQUENCY ULTRASOUND ON MINERALISATION IN LONG-TERM ODONTOBLAST-LIKE CULTURES

The dentine-pulp complex has the ability to regenerate dentine in response to injury by laying down reactionary dentine secreted by existing odontoblasts or by recruiting odontoblast-like cells derived from stem cells in the pulp that secrete reparative dentine (Smith *et al.* 1995a; Arana-Chavez and Massa 2004). The regeneration of dentine and dental pulp has been demonstrated in animal and *in vitro* studies (Nakashima 1994; Mooney *et al.* 1996; Iohara *et al.* 2004; El-Backly *et al.* 2008), which indicates the capacity of using tissue engineering concepts to regenerate the dentine-pulp complex. These studies will also enable the advancement of potential bioengineering strategies for tooth regeneration that will have a major societal and clinical impact for the management of dental disease (Smith 2003). The final step in dentine regeneration is mineralisation and studies have shown promotion of bone and cementum regeneration and enhanced mineralisation using LIPUS stimulation (Suzuki *et al.* 2009b; Rego *et al.* 2010). Thus LIPUS has been considered as a therapeutic tool for mineralised tissue repair including dentine repair and regeneration (Nakashima *et al.* 2003; El-Bialy 2007; Scheven *et al.* 2009b). Studies have shown that US is able to influence proliferation and VEGF expression in odontoblast-like cells that could encourage the normal process of healing (Scheven et al. 2009a). However, no study has yet demonstrated the direct effects of US on dentine mineralisation.

Reparative dentinogenesis is a complex process involving recruitment, proliferation and differentiation of host progenitor cells and involves intricate signalling networks, which are regulated by cytokines and growth factors (Murray *et al.* 2001; Smith 2003; Scheven et al. 2007). In the previous chapter, it was found that US stimulated

osteoblastic MC3T3-E1 cell migration in an *in vitro* wound healing assay, however US was not able to enhance wound repair in odontoblast-like cultures (**Chapter 6**), it is postulated here that US may be able to enhance dentine matrix production and mineralisation by odontoblasts thereby promoting dentine repair (Scheven *et al*, 2009). Thus the objective of this study was to examine the effect of US on *in vitro* mineralisation in long-term cultures of odontoblast-like MDPC-23 cells. For this purpose, the study utilised kHz US, which is used within dental clinical applications for the removal of dental plaque and root planing (Thilo *et al.* 1990). Different exposure times were assessed for their ability to enhance mineralisation and were chosen to resemble the treatment times that are currently in practice. The duration of US exposure from dental scaling varies between patients and is generally applied in short bursts with time of exposure per patient ranging from 1 to 13 minutes (Akesson *et al.* 2001). Shorter exposure times were therefore compared with a single 30 minute exposure, which was previously shown to be an effective treatment time to increase MDPC-23 cell proliferation (**Chapter 4**).

7.1 MINERALISATION ANALYSIS IN US-STIMULATED MDPC-23 CELLS

To determine whether US could enhance mineral production, MDPC-23 cells were stimulated with kHz US and cultured in mineralisation medium (MM) (**Section 2.6.1**) for 14 days until the formation of mineralised nodules became apparent in both control and US-stimulated groups. Calcium-rich deposits were then detected by alizarin red S (ARS) dye (**Section 2.6.2**). The results demonstrated an increased number of calcified nodules in cultures stimulated with US at all exposure times

compared with positive control cultures. Mineralisation was not observed in control or experimental cultures containing standard growth culture medium highlighting the importance of dexamethasone (dex), ascorbic acid (AA) and beta-glycerophosphate (β-GP) in the culture medium as additives for the promotion of osteogenic and odontogenic differentiation and mineralisation (Bellows *et al.* 1986; Narayanan *et al.* 2001)(**Figure 7-1**).

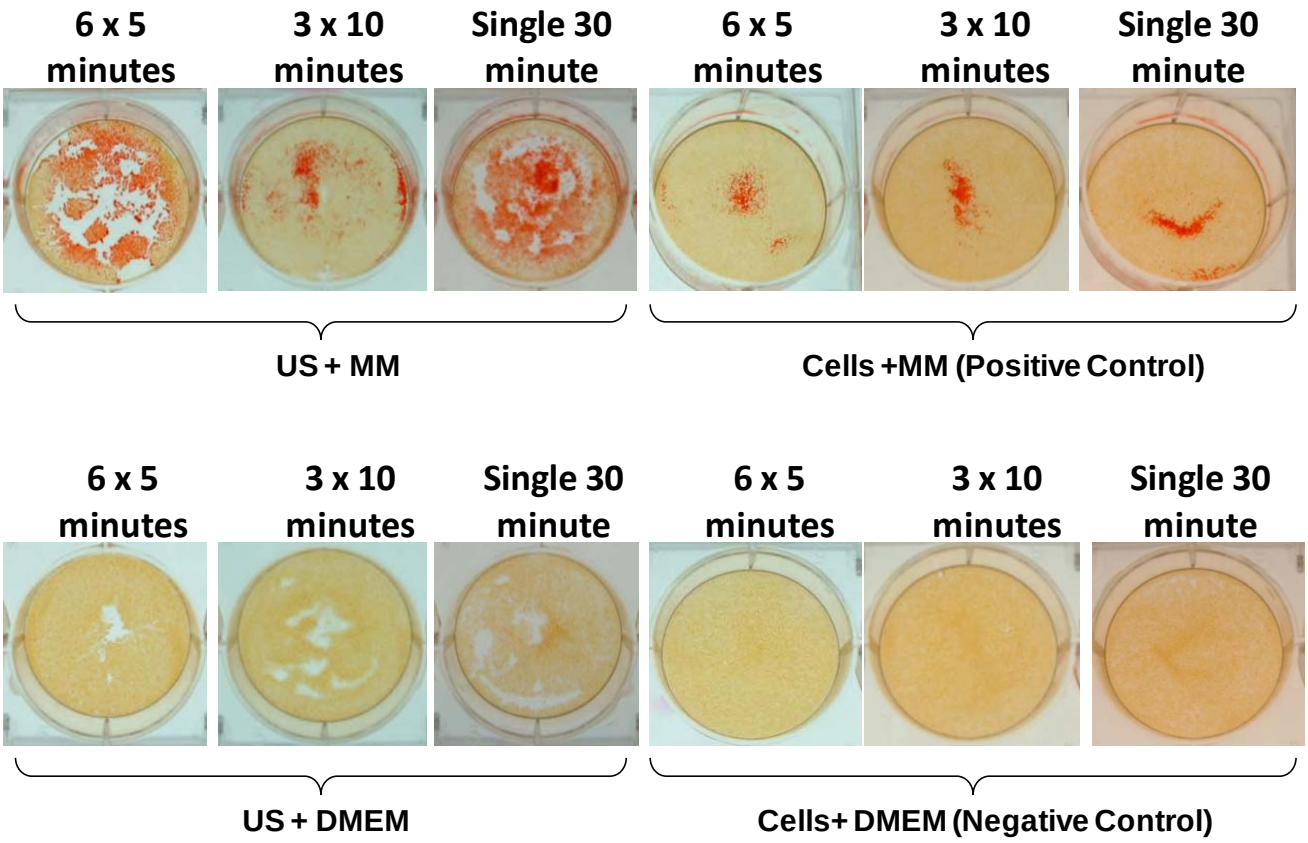


Figure 7-1 ARS staining of MDPC-23 cells cultured for 14 days in multi-well plates. Mineralisation as demonstrated by ARS was observed in MDPC-23 cells cultured in MM containing dex, AA and β-GP. Increased ARS staining was observed in 45 kHz US-stimulated groups cultured in MM compared with parallel cultures without US stimulation. Stimulation with a single 30 minute exposure to 45kHz US resulted in an visible higher deposition of mineral than stimulation with US for 6 daily 5 minute exposures or 3 daily 10 minute exposures. Each image is representative of triplicate cultures.

To quantify the level of ARS staining within the cultures, the stain was eluted and measured spectrophotometrically in a multi-well plate reader. To allow the comparison of mineral content between groups, the optical density was normalised to cell number in parallel cultures (**Figure 7-2**). Upon determining cell number on day 5, 9 and 14, it was evident MDPC-23 cells maintained in MM exhibited significantly increased cell number when compared with cells cultured in non-mineralising medium DMEM. In addition, MDPC-23 cell number was significantly reduced when exposed to kHz US at all treatment times applied. Moreover, US-stimulated cell number was comparable with unstimulated MDPC-23 cells in non-mineralising medium suggesting that US stimulation inhibited cell proliferation (**Figure 7-2**)

Quantification of mineralisation yielded the same staining pattern observed in **Figure 7-1** confirming the enhanced calcium deposition demonstrated in US-stimulated groups cultured in MM (**Figure 7-3**). Significantly higher mineral deposition was evident after a single 30-minute exposure and 3 daily 10-minute exposures with a single 30 minute exposure producing the highest level of mineral deposition (~ 6 times higher than positive control cultures followed by 3 x 10 minute exposures, which demonstrated mineral deposition ~ 2 times higher than positive control. Although cultures stimulated with 6 x 5 minute US exposures promoted enhanced mineral deposition, normalisation of results showed no significant increase in relative mineral deposition compared with positive control.

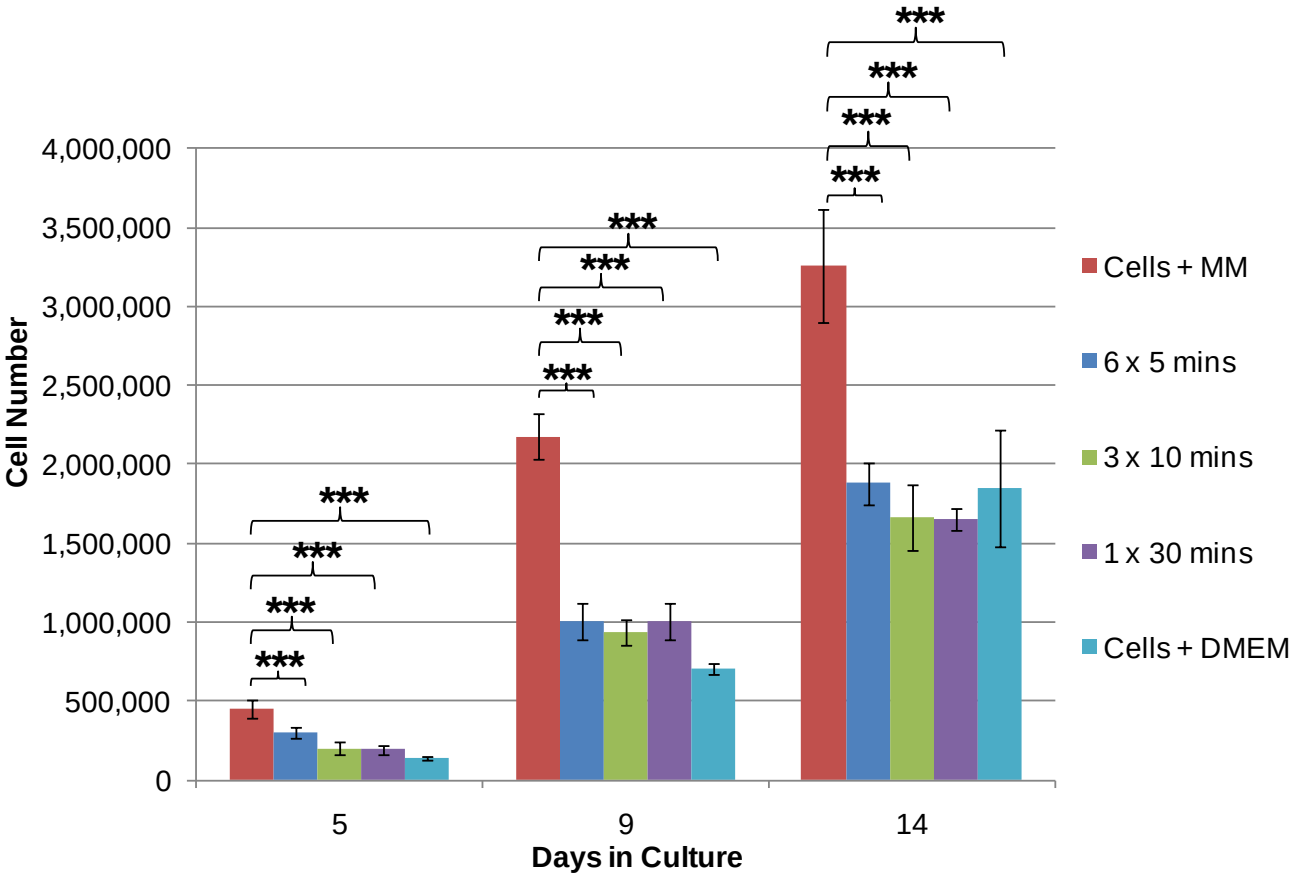
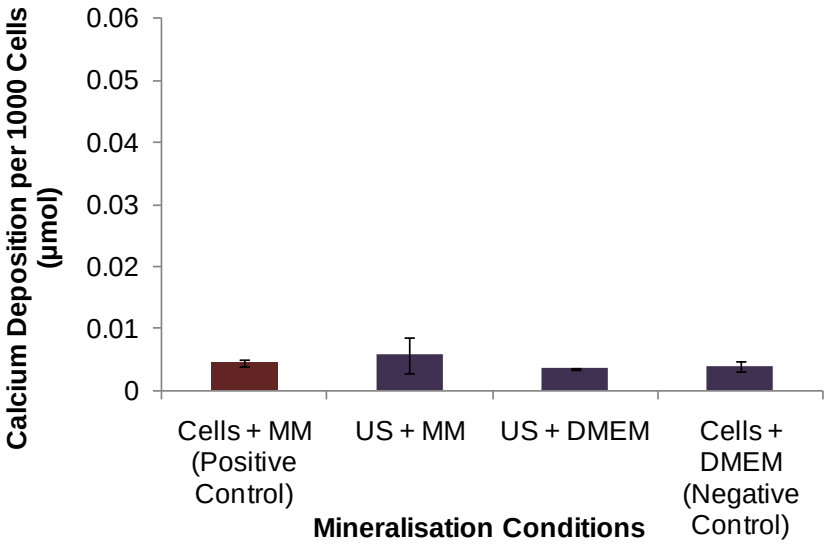
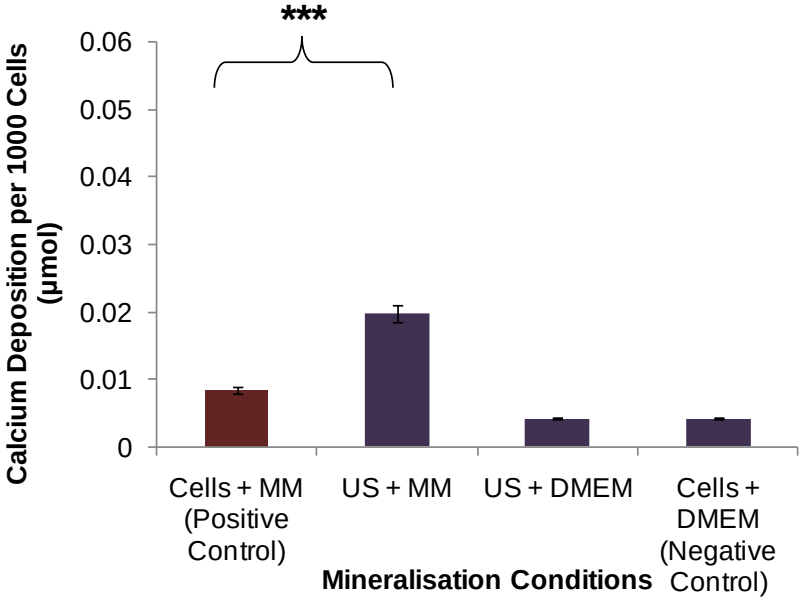


Figure 7-2 Influence of kHz US on MDPC-23 cell number in long-term cultures. Cell number of US-stimulated and control cells, as determined by cell count, was assessed at day 5, 9 and 14 of cell growth in MM. Data represents mean values from triplicate measurements ± SD. ***P<0.001 when compared with cells + MM group.

A


Calcium Deposition per 1000 Cells (µmol)
0.06
0.05
0.04
0.03
0.02
0.01
0
Cells + MM (Positive Control)
US + MM
US + DMEM
Cells + DMEM (Negative Control)
Mineralisation Conditions

B


***
Calcium Deposition per 1000 Cells (µmol)
0.06
0.05
0.04
0.03
0.02
0.01
0
Cells + MM (Positive Control)
US + MM
US + DMEM
Cells + DMEM (Negative Control)
Mineralisation Conditions

C

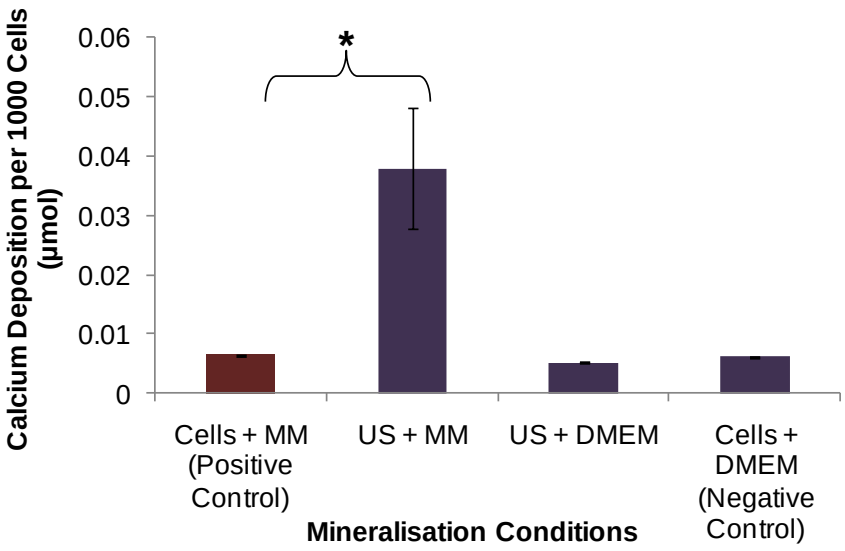
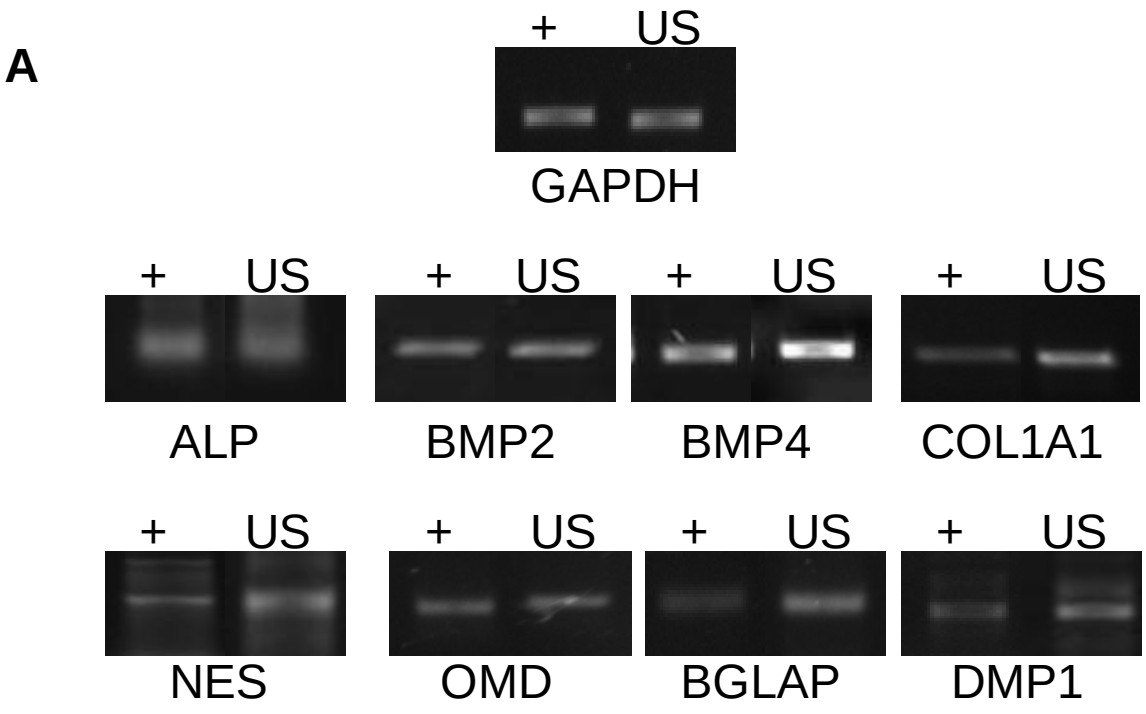

*
Calcium Deposition per 1000 Cells (µmol)
0.06
0.05
0.04
0.03
0.02
0.01
0
Cells + MM (Positive Control)
US + MM
US + DMEM
Cells + DMEM (Negative Control)
Mineralisation Conditions

Figure 7-3 Quantitative analysis of ARS staining in MDPC-23 cell cultures stimulated with 45kHz US for (**A**) 6 doses each at 5 minutes, (**B**) 3 doses each at 10 minutes and (**C**) a single 30 minute exposure. Ultrasound-stimulated MDPC-23 cells cultured in MM significantly increased mineral deposition when compared with parallel cultures without US stimulation with a single 30 minute US exposure. Data represents mean values from duplicate measurements ± SD normalised to cell number and corrected for background. *P<0.05 and ***P<0.001 when compared with controls.

7.2 MDPC-23 GENE EXPRESSION ANALYSIS FOLLOWING STIMULATION WITH LOW FREQUENCY ULTRASOUND

Gene expression was further analysed in MDPC-23 cells 12 days after initial US exposure for 30 minutes to confirm US-induced increased mineralisation demonstrated previously. Therefore, relative expression levels of mineralisation markers, dentine-related markers and relevant ECM markers using semi-quantitative RT-PCR were analysed. Agarose gel images obtained showed higher band intensities for alkaline phosphatase (ALP), bone morphogenetic protein 2 (BMP2), bone morphogenetic protein 4 (BMP4), collagen type I, alpha 1 (COL1A1), dentine matrix acidic phosphoprotein (DMP1), nestin (NES), osteoadherin (OMD) and osteocalcin (BGLAP) when compared with basal expression levels in unstimulated MDPC-23 cells cultured in MM (**Figure 7-4A**). Densitometric analysis of bands revealed significant elevated expression of COL1A1, OMD and NES. Enhanced expression was also notable for ALP, DMP-1 and BGLAP, which were elevated relative to those of control (**Figure 7-4B**). Relative expression of the heat shock 70kDa protein (HSPA), and transforming growth fact beta 1 (TGF-β1) were not substantially changed in cells subjected to US. Relative expression of the dentine sialophosphoprotein (DSPP) and heat shock 27kDa protein (HSPB) genes were not detected in control and experimental cultures.


A
+ US
GAPDH
+ US
ALP
+ US
BMP2
+ US
BMP4
+ US
COL1A1
+ US
NES
+ US
OMD
+ US
BGLAP
+ US
DMP1

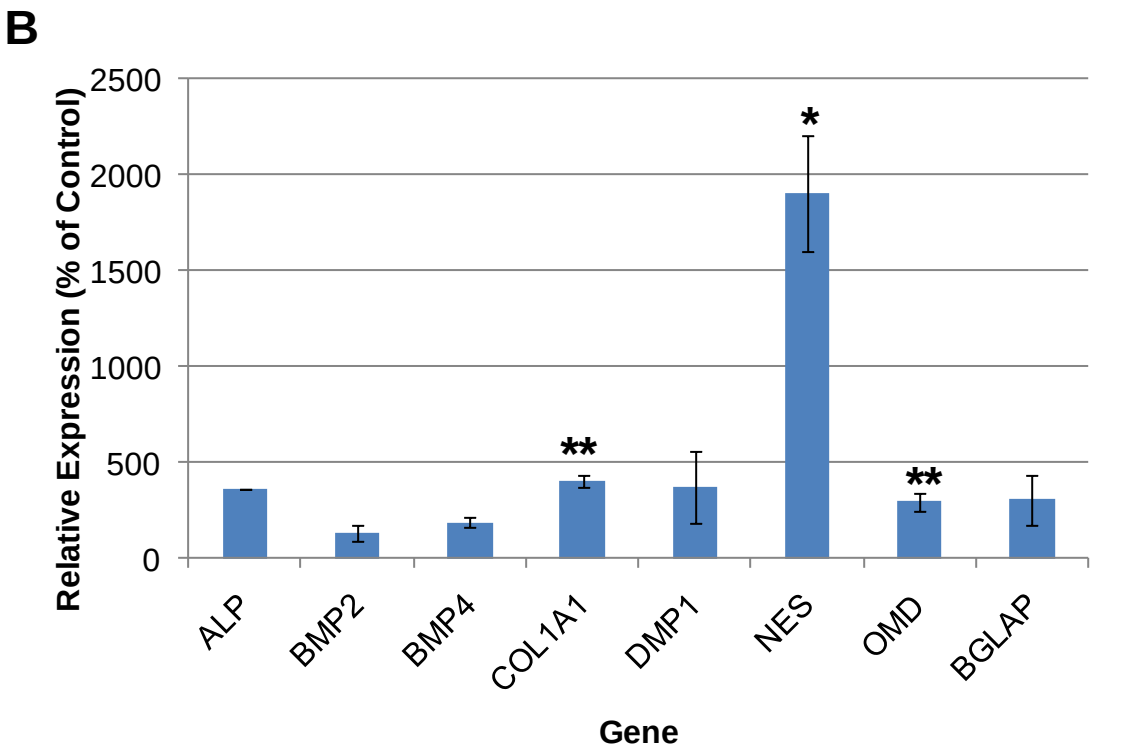

B
Relative Expression (% of Control)
2500
2000
1500
1000
500
0
*
**
**
ALP
BMP2
BMP4
COL1A1
DMP1
NES
OMD
BGLAP
Gene

Figure 7-4 Relative gene expression in odontoblast-like cells 14 days after a single 30 minute exposure to 45kHz US at an intensity of 25mW/cm^2. (**A**) Representative agarose gels showing differential gene expression analysis in positive control mineralising cells (+) and mineralising cells stimulated with US (US) demonstrating increased band intensities in 8 of the 15 marker genes assessed when compared with positive control. (**B**) Densitometric analysis of gel images shown were normalised against the GAPDH house-keeping gene control values. Agarose gels are representative images from triplicate samples and data is represented as percentage of positive controls (mean relative expression from duplicate measurements ± SE. *P<0.05 and **P<0.01 when compared with positive control.

7.3 DISCUSSION

Although information is not available for the effect of US on matrix mineralisation in odontoblast-like cells and its possible role in dentine repair, the responsiveness of other cell types, including osteoblasts and chondrocytes to LIPUS is well documented and may indicate that odontoblasts can respond to US. LIPUS is well established in accelerating bone fracture healing (Azuma *et al.* 2001; Martinez de Albornoz *et al.* 2011) as well as increasing bone mechanical strength (Chan *et al.* 2006; Kasturi and Adler 2011), which is facilitated by rapid production of mineralised matrix (Tsai *et al.* 1992; Sena *et al.* 2005; Unsworth *et al.* 2007; Suzuki *et al.* 2009a).

This present study demonstrates for the first time kHz US-induced enhancement of *in vitro* mineralisation by odontoblast-like cells. It should be noted that the results obtained from this present study may not be directly comparable with other studies involving LIPUS due to the differences in US frequency, intensities, exposure times and exposure system applied. Our results showed that a single exposure of US for 30 minutes resulted in the highest increase in mineral deposition, which was in fact the treatment regime that stimulated the highest cell proliferation as described in **Chapter 4**. However, in this present study, no increase in cell number was noted in US-stimulated cells maintained in MM suggesting that the enhancement of mineral deposition by US, which is indicative of osteogenic and odontogenic differentiation and subsequent mineralisation, was not mediated by cell proliferation. These findings are consistent with a number of similar *in vitro* studies for chondrocytes (Parvizi *et al.*

1999), human mesenchymal stem cells (Ebisawa *et al.* 2004) although no mechanism of action has been cited warranting further clarification.

Proliferation and differentiation of dental pulp cells resulting in production of reparative dentine-like tissue matrix involve processes that have yet not been fully understood. Even more poorly understood are the molecular mechanisms by which US is able to accelerate mineralisation *in vitro* however, some studies have indicated that LIPUS is able to enhance bone matrix production by enhancing different early and late osteoblastic differentiation markers and most notably enhancing ALP expression , which is considered a marker, albeit non-specific, for early cell differentiation and mineralisation (Leung *et al.* 2004; Unsworth *et al.* 2007; Suzuki *et al.* 2009b). The expression of ALP in this present study was notably increased as well as the expression of other differentiation markers that are related to mineralisation such as DMP1 and OMD (Lu *et al.* 2007; Rehn *et al.* 2008). It is conceivable that US may have promoted mineralisation by enhancing differentiation in MDPC-23 cells as opposed to direct effects of US on the functional activity of odontoblast-like cells.

Nestin is an odontoblast marker and thought to be an important marker of dentine repair and is expressed during limited periods through life such as during odontogenesis and in the repair of dentine (Terling *et al.* 1995; About *et al.* 2000; About and Mitsiadis 2001), thus, nestin is considered to be related to the differentiation toward odontoblasts and to the acquisition of the function to produce

dentine (McLachlan *et al.* 2003; Fujita *et al.* 2006). *In vitro* studies have shown that NES is upregulated in odontoblast-like cells that have produced mineralised matrix (About *et al.* 2000; About and Mitsiadis 2001). Indeed, NES was the most abundantly expressed gene analysed in US-stimulated mineralising cells and taken together with the upregulation in expression of COL1A, ALP and DMP1 expression, which have been shown to be up-regulated during the initial stages of the mineralisation process (Butler and Ritchie 1995; Honda *et al.* 2006), the findings from this present study may represent events in extracellular matrix formation before the initiation of mineral crystal formation. Furthermore, DMP1 has been shown to appear as an early marker for mineralisation and binding of DMP1 to collagen fibrils is thought to be important in mediating the onset of crystal formation (Orsini *et al.* 2008).

Rapid HSP gene activation has been reported in cells after various forms of stress including mechanical, toxic and heat and upon induction has a favourable impact on cell survival and repair. HSP activation is reported to be a possible mechanism by which LIPUS is able to accelerated soft tissue healing (Nussbaum and Locke 2007b; Kruse *et al.* 2008) and has also been detected after exposure to kHz US *in vitro* (Scheven et al. 2007). Interestingly, HSP was not detected in US-stimulated cells suggesting that the responses in this present study were not related to the onset of cell stress mechanisms. However, it is important to note that the results shown in this present study represent a snapshot of gene responses at a particular timeframe and therefore results should be interpreted with caution. This may be the reason why signalling molecules pertinent to dentine formation including TGF-β1 and BMP were not expressed or reduced expression was detected. To robustly determine the

molecular processes involved in US-stimulated mineralisation, it is necessary to assess the expression of gene markers and cell signalling processes at further time points. Nevertheless, these results demonstrate that US may be a potential tool for inducing and accelerating dentine formation and repair. The stimulation of mineral deposition and the upregulation of various key markers by US suggest a direct role in odontoblast differentiation and mineralisation.

In conclusion, the present investigation is first to demonstrate the stimulation and enhancement of mineral deposition by odontoblast-like cells stimulated by kHz US. Moreover, a single US exposure was the most effective at stimulating mineral production in MDPC-23 cell cultures, This result is in disagreement with the current LIPUS application regime that recommends using daily US exposures for accelerated healing of soft and hard tissues (Heckman *et al.* 1994; Kristiansen *et al.* 1997) and raises awareness of the uniquely different responses different cells may have to US frequency, intensity and exposure time. Importantly, these results highlight the possibility for US to be used as a restorative tool in dentistry to enhance dentine repair for the maintenance of tooth vitality (Smith 2003; Scheven *et al.* 2009b).

CHAPTER 8 GENERAL DISCUSSION AND CONCLUSION

8.1 SYSTEM FOR IN VITRO ULTRASOUND APPLICATION

This thesis described two methods for stimulating cells *in vitro*; the ultrasonic scaler system (**Chapter 3**) based on the method previously reported by (Sura et al. 2001); and the DuoSon set-up (**Chapter 4-7**) based on an exposure assembly reported by (Reher 1997; Scheven et al. 2007). Although using dental scalers to deliver US to cells *in vitro* is a unique and clinically relevant approach for assessing the US effects on dental cells, uncertainties regarding precise intensities and variables including multiple internal reflections, and standing wave formation may influence the intrinsic properties of the US wave making a precise mechanism of action difficult to determine. Furthermore, cellular induction of HSP was detected in cultures stimulated with US using dental scalers indicating that cellular stress may have been a confounding factor in the cellular responses demonstrated in that study (Scheven et al. 2007). Consequently, the DuoSon exposure system as described in this study, which was a calibrated and temperature-controlled system, enabled the application of a range of US frequencies and known intensities to dentally-derived cells *in vitro*. Data presented here indicated that the DuoSon assembly prevented thermal effects so that the results generated from this system may be largely considered non-thermal responses.

Formation of standing waves were minimised by using an anti-reflective chamber as well as via direct US exposure to the cells, a technique employed by other studies (Loch *et al.* 1971; Reher *et al.* 1998; McCormick *et al.* 2006). To further reduce the formation of standing waves, degassed water may also be used in the DuoSon

experimental system to prevent secondary reflections from any oxygen bubbles present in the water bath, which may ultimately alter the overall power of the US wave delivered (Kaufman and Miller 1978; Church and Miller 1983).

The relevance of *in vitro* studies to the clinical setting is unclear however these US application systems allow the investigation of the mechanistic and cellular responses to US with exceptional sensitivity enabling accurate delivery of US to cells at different and well-characterised US parameters that cannot be achieved *in vivo* (Dinno *et al.* 1989). Odontoblast-like and osteoblast-like cells were utilised to study cellular responses to US and both have proven its usefulness and applicability for dentine repair (Sun *et al.* 1998; Simon *et al.* 2010) and osteogenesis, respectively (Wang *et al.* 1999; Hoemann *et al.* 2009). Whilst only some phenotypic characteristics of odontoblasts (Hanks *et al.* 1998b) and osteoblasts (Quarles *et al.* 1992; Stein and Lian 1993) are maintained in these cell lines, they offer easily accessible and readily available models that are valuable for studying the cell behaviour in response to US.

8.2 ULTRASOUND PARAMETERS FOR MAXIMAL STIMULATION OF ODONTOBLAST AND OSTEOBLAST-LIKE CELLS.

Currently, there are no clear guidelines for the application of US *in vitro* or *in vivo*. A review of the literature reveals a broad range of treatment settings and US delivery methods, which differ in frequency, acoustic wave form, intensity, treatment times and number of treatments required for efficacy (Srbely 2008). Furthermore, different cells respond differently to these US parameters. For example, daily exposure to

LIPUS at 1.5MHz and 30mW/cm^2 for 20 minutes did not alter cell number in MC3T3-E1 cells (Unsworth *et al.* 2007) but significantly increased chondrocyte cell number (Takeuchi *et al.* 2008). In this study, 15 minutes of US at all frequencies applied did not alter MDPC-23 cell number however after exposure to the same US parameters, MC3T3-E1 cell number was significantly increased (**Chapter 4**). Therefore among different researchers, not only do the US parameters for maximal stimulation vary (Doan *et al.* 1999; Li *et al.* 2002; Hayton *et al.* 2005), the reported cellular responses to US also vary between different research groups. Due to the inconsistencies in US treatment results, there is insufficient data to generate scientifically sound recommendations for the promotion of tissue repair by US stimulation and may contribute to the wide range of contrasting cellular effects following US stimulation within the literature therefore reported effects such as US-induced accelerated proliferation remain controversial. A variety of systems are employed for exposing cells to US ranging from elaborate arrangements with temperature-controlled, degassed tanks to simple transducers attached to culture plastic however, disturbances to US transduction, which can ultimately affect how cells behave in response to the US signal. As such, the data generated in this thesis cannot be directly comparable to other studies due to the differences in parameters and exposure systems applied.

This study is the first to compare cellular responses to different US frequencies, continuous and pulsed acoustic waveforms, treatment times and the effect of single and multiple US doses in odontoblast-like and osteoblast-like cells. Data indicated that the application of US using the DuoSon exposure system identified a delivery

approach which was able to enhance cell proliferation in monolayers cultures (**Chapter 4**) enhance wound repair in osteoblast-like cells by up-regulating cell proliferation and migration (**Chapter 6**) and enhance mineral deposition in MDPC-23 cells (**Chapter 7**). In this thesis, pulsed US frequency of 1MHz was most suitable for MDPC-23 cell proliferation whereas continuous US at a frequency of 45kHz was best suited for MC3T3-E1 cell proliferation. 45kHz US was also demonstrated to stimulate bone formation that was equal to or higher than traditional 1MHz US (Reher 1997; Reher *et al.* 1998). Taken together, it is interesting to speculate that stimulation of cells with 45kHz US is comparable with standard 1MHz US and has additional advantages, such as better tissue penetration and a wider exposure area.

Combined 45kHz and 1MHz frequency US, which previously has yet to be studied for its biological responses, is unique to the DuoSon device that is hypothesised to enhance wound healing in chronic injuries for both superficial and deeper tissue structures (SRA Developments, UK). The data in this thesis demonstrated that stimulation with combined frequency US elicited cell responses comparable with those stimulated by either 45kHz or 1 MHz at optimum exposure times. Furthermore, stimulation of osteoblast-like cells by combined frequency US resulted in the highest scratch wound healing rate thereby providing supporting data for the use of this novel modality for wound repair. However, combined frequency US resulted in severe loss in cell viability at increased exposure times (**Chapter 4**) and may therefore be recommended for shorter exposure times only.

Here in this thesis, comparison was made between 45kHz, 1MHz and combined frequency US (**Chapter 4, 5, 6**), however, the frequency assessed in this study differed in acoustic waveform US intensity and US power output that will ultimately change the behaviour of the acoustic wave and affect cell responses. To find the optimum parameters for peak proliferation response in odontoblast-like and osteoblast-like cells, the number of independent variables needs to be reduced and the dynamic between a specific parameter and cell response will need to be independently studied. The data from this thesis however provides a range of parameter settings for increased odontoblast-like and osteoblast-like cell proliferation in monolayers cultures, which, in the case of osteoblasts, differs from what is generally reported in the literature (Dyson and Brookes 1983; Yang *et al.* 1996; Harle *et al.* 2001a). The duration of US stimulation and the multiple dose response has been studied to a lesser extent (Tsai *et al.* 1992; Chan *et al.* 2006).

In this study, duration of US exposure had a significant impact on cell proliferation (**Chapter 4**) and mineral deposition in MDPC-23 cells (**Chapter 7**). A lower and upper threshold was established to which odontoblast-like and osteoblast-like cells responded to US-stimulation (**Chapter 4**), which suggests that these cells differ in their sensitivity to a particular range in US dose. On assessing the length of US exposure on cell proliferation, doubling the exposure time did not elicit any discernable differences from a shorter or a single exposure demonstrating a habituation response. Indeed, it has been demonstrated that cells tend to become less sensitive to chronic US exposures (Waldman *et al.* 2003) and it is well established that mechanosensitive cells, such as osteoblasts, adapt to mechanical

stress and their sensitivity to mechanical stress decreases after longer stimulations (Turner 1998; Saxon *et al.* 2005; Schriefer *et al.* 2005).

While there are general recommendations for US treatment the possibility exists that clinical treatment protocols commonly employed for US are not sufficient for therapeutic efficacy (Hashish *et al.* 1986; Ebenbichler *et al.* 1999; Robertson and Baker 2001). For *in vitro* research, it may be more accurate to define optimum parameters according to the exposure system that is being employed rather than the biological response that is being altered by US stimulation and it is conceivable that US-induced biological effects stated in the literature are most likely to be dependent on the experimental system applied. It is likely that the cell responses described in this thesis will differ from those *in vivo* due to US attenuation of the surrounding structures resulting in energy loss in the US beam. It is conceivable therefore that higher intensities and powers are required *in vivo* for similar *in vitro* effects. However, by studying the biological effects of US *in vitro,* a range of US treatment modalities can be directly assayed and screened, which allows better identification of the molecular and cellular processes underlying the actions of US.

8.3 POTENTIAL USE OF US FOR DENTAL TISSUE REPAIR

Low frequency US for the repair of dental tissues has gained interest due to the well-established effects of LIPUS for bone fracture healing and soft tissue repair (Flemming and Cullum 2000; Busse *et al.* 2002). As such, the use of US for dental tissue repair is now being explored and has been applied to stimulate bone healing

and formation during mandibular osteogenesis (El-Bialy *et al.* 2002), stimulate dental tissue formation and enhanced teeth eruption (El-Bialy *et al.* 2003a) as well as being very effective for enhancing periodontal wound healing (Ikai *et al.* 2008; Shiraishi *et al.* 2011). Furthermore, research is being undertaken to develop an intraoral US device to encourage neoangiogenesis and tissue matrix formation for tissue healing by directed tissue stimulation (Woon Tiong *et al.* 2008).

For the first time, data is now presented here that provides evidence for the potential therapeutic benefits of US to odontoblast-like cells. In this study, stimulation and enhancement of mineral deposition by odontoblast-like cells was achieved by US stimulation at 45kHz and molecular analyses implicated US to have a direct role in odontoblast differentiation and mineralisation (**Chapter 7**). To the author's knowledge, this is the first study to indicate that US stimulation directly affects odontoblast-mediated mineral deposition. Indeed LIPUS has been previously shown to initiate osteoblast differentiation and enhance mineralisation *in vitro* (Takayama *et al.* 2007; Suzuki *et al.* 2009b; Angle *et al.* 2011).

It is tempting to speculate, when taken with the results presented in this thesis, that operation of dental ultrasonic scalers at 35kHz during clinical procedures may induce changes to cell behaviour whether it is through the influence of thermal transduction (**Chapter 3**) or mechanical vibrations or both (Nishimura *et al.* 2008). If US stimulation was to be directed at the dentine-pulp complex or alveolar bone, it is conceivable that US may be able to initiate or enhance regenerative events by

enhancing proliferation (**Chapter 4**), encouraging angiogenic responses (**Chapter 3**) and by increasing the production of mineral (**Chapter 7**) in odontoblasts and osteoblasts. Ultrasound therefore has the potential to be developed as a non-invasive, regenerative tool for dental tissue healing (Scheven *et al.* 2009b).

Significant studies are required in order to understand how US may deliver therapeutic effects. The variation in observed effects due to the use of different US parameters and delivery systems makes determination of the precise mechanism of action difficult. However; a major theory regarding US transduction is through the mechano-sensitisation of integrins (Yang *et al.* 2005; Tang *et al.* 2006). To explore this further, an attempt to visualise US-induced changes in the actin cytoskeleton was made (**Chapter 5**). The actin cytoskeleton is reportedly key to regulating the adhesive function of integrins and is reported to be influenced by mechanical stress (Delon and Brown 2007). Although US was able to influence cell shape (Altland *et al.* 2004), no alterations to the actin cytoskeleton were demonstrated, which suggests that US transduction in odontoblast-like and osteoblast-like cells was not integrin-mediated. However, the Wnt/β-catenin pathway may play a possible role in US transduction (**Chapter 3** and **Chapter 5**). Interestingly, Wnt activation has been suggested as a possible US signal transduction pathway in several other studies using different cell types (Takeuchi *et al.* 2008; Olkku *et al.* 2010). Further investigations for US-induced Wnt-activity could examine the role of the PI3/Akt pathway, which is known to have functional interaction with Wnt/β-catenin pathway and thus US may increase intracellular β-catenin levels, as demonstrated in **Chapter 3** and **Chapter 5**, via the PI3/Akt pathway (Naruse *et al.* 2003; Takeuchi *et al.* 2008).

Furthermore, this pathway is associated with various functions such as cell survival, proliferation, motility, control of cell size and metabolism (Downward 2004; Gustin *et al.* 2006); many of which have been demonstrated to be enhanced by US in this study.

8.4 SUMMARY AND CONCLUSION

It is currently an exciting era for dental and craniofacial research due to the significant progress made towards defining the processes for tooth development and repair. Currently, the major focus of study is on the biological regeneration of oral tissues and due to the manipulation of 21st century advances in stem cell biology and biotechnology, biological solutions to dental problems such as tissue-engineered bone and dentine to replace injured tissue is now becoming a reality. The novel data presented in this thesis has demonstrated that low frequency US emitted via dental scalers, which are routinely used for the management of periodontal disease, may also have a biological effect on oral tissues. Moreover, the data here suggests that US may be exploited to promote dentine repair supporting the hypothesis that US may be a potential effective tool to stimulate dentine formation (Scheven *et al.* 2009b). This thesis has demonstrated that US at a frequency comparable to that of US emitted via dental scalers can enhance mineral deposition *in vitro* and therefore US stimulation of odontoblasts may have the potential to promote tooth repair by inducing reparative dentinogenesis. Furthermore, US has been demonstrated in this study to increase osteoblast-like cell proliferation, as well as enhance *in vitro* wound repair by increasing cell migration. It is conceivable that low frequency US may also be suitable as a non-invasive tool for accelerated bone regeneration and osseous integration in mineralisaed oral and maxillofacial surgical procedures. However, much work is still required to fully understand the mechanism of intracellular US signal transduction as well as to determine a treatment regime that will induce, promote or enhance positive effects of US within dentally-derived cells. With better understanding and further research, US could form a powerful tool in future clinical

dental therapies by promoting long-term tooth vitality, facilitated bone repair and ultimately improve quality of life (Scheven *et al.* 2009b).

8.5 FURTHER STUDIES

These studies have provided an insight into: 1) the mechanisms by which US exerts its biological effects in bone and tooth cells, 2) possible signalling transduction pathways by which US converts extracellular mechanical waves to intracellular biochemical signals and 3) the potential benefits for US in dental tissue regeneration. However, these studies have also raised questions and uncertainties that remained unanswered. The following account outlines studies that could be performed to address these questions.

SYSTEM FOR IN VITRO ULTRASOUND APPLICATION

The ultrasonic scaler device (**Chapter 3**) provided a unique and clinically relevant system for assessing the response of dental cells to low frequency US. However, due to the design of the ultrasonic tips, US delivery was limited to cells in suspension that would ultimately reduce the validity of the experiment. To overcome this problem, a dental ultrasonic tip could be designed that would fully immerse into a 6-well plate containing adherent cells. These experiments can then be performed under temperature control using the heated anti-reflection chamber as described in **Section 2.1.2**.

CELLULAR RESPONSES TO ULTRASOUND

The cellular effects of low frequency, low intensity US is generally considered to be non-thermal in origin (Dyson 1982). Although the non-thermal effects of US were successfully isolated using the DuoSon US delivery system and the thermal effects were minimised (**Chapter 3**), it may be incorrect to assume that the results from this thesis had not been influenced by small temperature fluctuations. It is therefore more accurate to assume that the non-thermal effects of US will always be accompanied by temperature changes and that the thermal effects of US cannot be eliminated completely (ter Haar 1999). To investigate the influence of small fluctuations of temperature on cellular response, further studies could include parallel experiments simulated with temperature only.

The US exposure model for this thesis utilises cell lines to study the responses to US stimulation and caution is required in the interpretation of the results since the cell responses may differ between and among species. For example, it has been suggested that the rat dental pulp is capable of a stronger reparative response following injury than the human pulp (Smith 2002). Future work should proceed using a more clinically relevant biological system such as an *ex vivo* tooth slice organ model (Murray *et al.* 2000). This will provide a useful insight into how US propagates through different dental tissue structures, how the dental pulp may respond to US and its role in reparative dentinogenesis and dentine regeneration.

POTENTIAL USE OF ULTRASOUND FOR DENTAL TISSUE REPAIR

Research into dentine repair by effective delivery of bioactive molecules is currently a major focus in dental research (Nakashima 1994; Smith *et al.* 1994; Smith *et al.* 1995a; Hu *et al.* 1998) and several molecules have been shown to influence the healing process by stimulating the recruitment and differentiation of pulp progenitors including BMPs, TGFβ, VEGF (Nomi *et al.* 2006; Soden *et al.* 2009; Yang *et al.* 2009). In this thesis, low frequency US has the potential to stimulate tooth repair by induction of VEGF involving cellular responses such as cell proliferation and neo-angiogenesis (**Chapter 3**). Furthermore, low frequency US may potentially be used to deliver these bioactive molecules to the site of injury by US-mediated gene transfer to induce reparative dentine (Nakashima *et al.* 2004).

REFERENCES

About I, Bottero MJ, de Denato P, Camps J, Franquin JC, Mitsiadis TA (2000) Human Dentin Production in Vitro. Exp Cell Res 258(1):**33-41**

About I, Mitsiadis TA (2001) Molecular Aspects of Tooth Pathogenesis and Repair: In Vivo and in Vitro Models. Adv Dent Res 15:**59-62**

Akesson I, Balogh I, Skerfving S (2001) Self-Reported and Measured Time of Vibration Exposure at Ultrasonic Scaling in Dental Hygienists. Appl Ergon 32(1):**47-51**

Akita K, Francis-West P, Vargesson N (1996) The Ectodermal Control in Chick Limb Development: Wnt-7a, Shh, Bmp-2 and Bmp-4 Expression and the Effect of Fgf-4 on Gene Expression. Mech Dev 60(2):**127-137**

Altland OD, Dalecki D, Suchkova VN, Francis CW (2004) Low-Intensity Ultrasound Increases Endothelial Cell Nitric Oxide Synthase Activity and Nitric Oxide Synthesis. J Thromb Haemost 2(4):**637-643**

Alvarenga EC, Rodrigues R, Caricati-Neto A, Silva-Filho FC, Paredes-Gamero EJ, Ferreira AT (2010) Low-Intensity Pulsed Ultrasound-Dependent Osteoblast Proliferation Occurs by Via Activation of the P2y Receptor: Role of the P2y1 Receptor. Bone 46(2):**355-362**

Amano T, Muramatsu T, Amemiya K, Kubo K, Shimono M (2006) Responses of Rat Pulp Cells to Heat Stress in Vitro. J Dent Res 85(5):**432-435**

Angle SR, Sena K, Sumner DR, Virdi AS (2011) Osteogenic Differentiation of Rat Bone Marrow Stromal Cells by Various Intensities of Low-Intensity Pulsed Ultrasound. Ultrasonics 51(3):**281-288**

Aplin AE, Howe A, Alahari SK, Juliano RL (1998) Signal Transduction and Signal Modulation by Cell Adhesion Receptors: The Role of Integrins, Cadherins, Immunoglobulin-Cell Adhesion Molecules, and Selectins. Pharmacol Rev 50(2):**197-263**

Arana-Chavez VE, Massa LF (2004) Odontoblasts: The Cells Forming and Maintaining Dentine. Int J Biochem Cell Biol 36(8):**1367-1373**

Arya R, Mallik M, Lakhotia SC (2007) Heat Shock Genes - Integrating Cell Survival and Death. J Biosci 32(3):**595-610**

Atrizadeh F, Kennedy J, Zander H (1971) Ankylosis of Teeth Following Thermal Injury. J Periodontal Res 6(3):**159-167**

Azuma Y, Ito M, Harada Y, Takagi H, Ohta T, Jingushi S (2001) Low-Intensity Pulsed Ultrasound Accelerates Rat Femoral Fracture Healing by Acting on the Various Cellular Reactions in the Fracture Callus. J Bone Miner Res 16(4):**671-680**

Baker KG, Robertson VJ, Duck FA (2001) A Review of Therapeutic Ultrasound: Biophysical Effects. Phys Ther 81(7):**1351-1358**

Barnett SB, Rott HD, ter Haar GR, Ziskin MC, Maeda K (1997) The Sensitivity of Biological Tissue to Ultrasound. Ultrasound Med Biol 23(6):**805-812**

Barnett SB, ter Haar GR, Ziskin MC, Nyborg WL, Maeda K, Bang J (1994) Current Status of Research on Biophysical Effects of Ultrasound. Ultrasound Med Biol 20(3):**205-218**

Bashutski JD, Wang HL (2009) Periodontal and Endodontic Regeneration. J Endod 35(3):**321-328**

Baskin L, Howard PS, Macarak E (1993) Effect of Mechanical Forces on Extracellular Matrix Synthesis by Bovine Urethral Fibroblasts in Vitro. J Urol 150(2 Pt 2):**637-641**

Basso O, Pike JM (1998) The Effect of Low Frequency, Long-Wave Ultrasound Therapy on Joint Mobility and Rehabilitation after Wrist Fracture. J Hand Surg Br 23(1):**136-139**

Baume LJ (1980) The Biology of Pulp and Dentine. A Historic, Terminologic-Taxonomic, Histologic-Biochemical, Embryonic and Clinical Survey. Monogr Oral Sci 8:**1-220**

Behari J, Singh S (1981) Ultrasound Propagation in 'in Vivo' Bone. Ultrasonics 19(2):**87-90**

Bellows CG, Aubin JE, Heersche JN, Antosz ME (1986) Mineralized Bone Nodules Formed in Vitro from Enzymatically Released Rat Calvaria Cell Populations. Calcif Tissue Int 38(3):**143-154**

Berkovitz BKB, Holland GR, Moxham BJ (2005) Oral Anatomy, Histology and Embryology, 3rd edn. Mosby International Limited, Edinburgh, UK. **p102, 103, 104, 125, 137, 141, 142, 326**

Bernard GW (1972) Ultrastructural Observations of Initial Calcification in Dentine and Enamel. J Ultrastruct Res 41(1):**1-17**

Bouchet-marquis Cd, Zuber Bt, Glynn A-M, Eltsov M, Grabenbauer M, Goldie KN, Thomas D, Frangakis AS, Dubochet J, ChrÃ©tien D (2007) Visualization of Cell Microtubules in Their Native State. Biology of the Cell 099(1):**45-53**

Bradnock B (1994) Longwave Ultrasound in Soft Tissue Injury. International Journal of Sports and Medicine in Soft Tissue Trauma 6(1):**6-7**

Breuing KH, Bayer L, Neuwalder J, Orgill DP (2005) Early Experience Using Low-Frequency Ultrasound in Chronic Wounds. Ann Plast Surg 55(2):**183-187**

Brine EJ, Marretta SM, Pijanowski GJ, Siegel AM (2000) Comparison of the Effects of Four Different Power Scalers on Enamel Tooth Surface in the Dog. J Vet Dent 17(1):**17-21**

Buchtala V (1950) [Present State of Ultrasound Therapy]. Dia Med 22(70):**2944-2950**

Busse JW, Bhandari M, Kulkarni AV, Tunks E (2002) The Effect of Low-Intensity Pulsed Ultrasound Therapy on Time to Fracture Healing: A Meta-Analysis. CMAJ : Canadian Medical Association journal = journal de l'Association medicale canadienne 166(4):**437-441**

Butler WT, Ritchie H (1995) The Nature and Functional Significance of Dentin Extracellular Matrix Proteins. Int J Dev Biol 39(1):**169-179**

Caldwell GM, Jones C, Gensberg K, Jan S, Hardy RG, Byrd P, Chughtai S, Wallis Y, Matthews GM, Morton DG (2004) The Wnt Antagonist Sfrp1 in Colorectal Tumorigenesis. Cancer Res 64(3):**883-888**

Casarotto RA, Adamowski JC, Fallopa F, Bacanelli F (2004) Coupling Agents in Therapeutic Ultrasound: Acoustic and Thermal Behavior. Arch Phys Med Rehabil 85(1):**162-165**

Chan CW, Qin L, Lee KM, Zhang M, Cheng JC, Leung KS (2006) Low Intensity Pulsed Ultrasound Accelerated Bone Remodeling During Consolidation Stage of Distraction Osteogenesis. J Orthop Res 24(2):**263-270**

Chang WH, Sun JS, Chang SP, Lin JC (2002) Study of Thermal Effects of Ultrasound Stimulation on Fracture Healing. Bioelectromagnetics 23(4):**256-263**

Chiba M, Mitani H (2004) Cytoskeletal Changes and the System of Regulation of Alkaline Phosphatase Activity in Human Periodontal Ligament Cells Induced by Mechanical Stress. Cell Biochem Funct 22(4):**249-256**

Church CC, Miller MW (1983) The Kinetics and Mechanics of Ultrasonically-Induced Cell Lysis Produced by Non-Trapped Bubbles in a Rotating Culture Tube. Ultrasound Med Biol 9(4):**385-393**

Claes L, Willie B (2007) The Enhancement of Bone Regeneration by Ultrasound. Prog Biophys Mol Biol 93(1-3):**384-398**

Clevers H (2006) Wnt/[Beta]-Catenin Signaling in Development and Disease. Cell 127(3):**469-480**

Cobb JP, Hotchkiss RS, Karl IE, Buchman TG (1996) Mechanisms of Cell Injury and Death. Br J Anaesth 77(1):**3-10**

Connor CW, Hynynen K (2004) Patterns of Thermal Deposition in the Skull During Transcranial Focused Ultrasound Surgery. IEEE Trans Biomed Eng 51(10):**1693-1706**

Coomber BL, Gotlieb AI (1990) In Vitro Endothelial Wound Repair. Interaction of Cell Migration and Proliferation. Arteriosclerosis 10(2):**215-222**

Cooper JA (1987) Effects of Cytochalasin and Phalloidin on Actin. J Cell Biol 105(4):**1473-1478**

Cooper KE (2002) Some Historical Perspectives on Thermoregulation. J Appl Physiol 92(4):**1717-1724**

Cooper PR, Takahashi Y, Graham LW, Simon S, Imazato S, Smith AJ (2010) Inflammation-Regeneration Interplay in the Dentine-Pulp Complex. J Dent 38(9):**687-697**

Couve E (1986) Ultrastructural Changes During the Life Cycle of Human Odontoblasts. Arch Oral Biol 31(10):**643-651**

Dalla-Bona DA, Tanaka E, Inubushi T, Oka H, Ohta A, Okada H, Miyauchi M, Takata T, Tanne K (2008) Cementoblast Response to Low- and High-Intensity Ultrasound. Arch Oral Biol 53(4):**318-323**

Delon I, Brown NH (2007) Integrins and the Actin Cytoskeleton. Curr Opin Cell Biol 19(1):**43-50**

Deng J, Xia W, Miller SA, Wen Y, Wang HY, Hung MC (2004) Crossregulation of Nf-Kappab by the Apc/Gsk-3beta/Beta-Catenin Pathway. Mol Carcinog 39(3):**139-146**

Denier A (1946) Ultrasonoscope in Medicine. CR Acad Sci Paris 22:**785-786**

Derringer KA, Linden RWA (2004) Vascular Endothelial Growth Factor, Fibroblast Growth Factor 2, Platelet Derived Growth Factor and Transforming Growth Factor Beta Released in Human Dental Pulp Following Orthodontic Force. Archives of Oral Biology 49(8):**631**

Dhopatkar AA, Sloan AJ, Rock WP, Cooper PR, Smith AJ (2005) British Orthodontic Society, Chapman Prize Winner 2003. A Novel in Vitro Culture Model to Investigate the Reaction of the Dentine-Pulp Complex to Orthodontic Force. J Orthod 32(2):**122-132**

Dimitriou R, Babis GC (2007) Biomaterial Osseointegration Enhancement with Biophysical Stimulation. J Musculoskelet Neuronal Interact 7(3):**253-265**

Dinno MA, Dyson M, Young SR, Mortimer AJ, Hart J, Crum LA (1989) The Significance of Membrane Changes in the Safe and Effective Use of Therapeutic and Diagnostic Ultrasound. Phys Med Biol 34(11):**1543-1552**

Doan N, Reher P, Meghji S, Harris M (1999) In Vitro Effects of Therapeutic Ultrasound on Cell Proliferation, Protein Synthesis, and Cytokine Production by Human Fibroblasts, Osteoblasts, and Monocytes. J Oral Maxillofac Surg 57(4):**409-419; discussion 420**

Dobie K, Smith G, Sloan AJ, Smith AJ (2002) Effects of Alginate Hydrogels and Tgf-Beta 1 on Human Dental Pulp Repair in Vitro. Connect Tissue Res 43(2-3):**387-390**

Doblhoff-Dier O, Gaida T, Katinger H, Burger W, Groschl M, Benes E (1994) A Novel Ultrasonic Resonance Field Device for the Retention of Animal Cells. Biotechnol Prog 10(4):**428-432**

Downward J (2004) Pi 3-Kinase, Akt and Cell Survival. Semin Cell Dev Biol 15(2):**177-182**

Draper BK, Komurasaki T, Davidson MK, Nanney LB (2003) Epiregulin Is More Potent Than Egf or Tgfalpha in Promoting in Vitro Wound Closure Due to Enhanced Erk/Mapk Activation. J Cell Biochem 89(6):**1126-1137**

Du C, Moradian-Oldak J (2006) Tooth Regeneration: Challenges and Opportunities for Biomedical Material Research. Biomed Mater 1(1):**R10-17**

Duarte LR (1983) The Stimulation of Bone Growth by Ultrasound. Arch Orthop Trauma Surg 101(3):**153-159**

Dufourcq P, Couffinhal T, Ezan J, Barandon L, Moreau C, Daret D, Duplaa C (2002) Frza, a Secreted Frizzled Related Protein, Induced Angiogenic Response. Circulation 106(24):**3097-3103**

Dyson M (1982) Non-Thermal Cellular Effects of Ultrasound. Br J Cancer Suppl 5:**165-171**

Dyson M, Brookes M (1983) Stimulation of Bone Repair by Ultrasound. Ultrasound Med Biol Suppl 2:**61-66**

Dyson M, Suckling J (1978) Stimulation of Tissue Repair by Ultrasound: A Survey of the Mechanisms Involved. Physiotherapy 64(4):**105-108**

Ebenbichler GR, Erdogmus CB, Resch KL, Funovics MA, Kainberger F, Barisani G, Aringer M, Nicolakis P, Wiesinger GF, Baghestanian M, Preisinger E, Fialka-Moser V (1999) Ultrasound Therapy for Calcific Tendinitis of the Shoulder. N Engl J Med 340(20):**1533-1538**

Ebisawa K, Hata K, Okada K, Kimata K, Ueda M, Torii S, Watanabe H (2004) Ultrasound Enhances Transforming Growth Factor Beta-Mediated Chondrocyte Differentiation of Human Mesenchymal Stem Cells. Tissue Eng 10(5-6):**921-929**

Efanov OI (1981) [Effect of Low-Intensity Ultrasound on the Microcirculation in Periodontosis]. Vopr Kurortol Fizioter Lech Fiz Kult(3):**5-8**

El-Backly RM, Massoud AG, El-Badry AM, Sherif RA, Marei MK (2008) Regeneration of Dentine/Pulp-Like Tissue Using a Dental Pulp Stem Cell/Poly(Lactic-Co-Glycolic) Acid Scaffold Construct in New Zealand White Rabbits. Aust Endod J 34(2):**52-67**

El-Bialy T (2007) Therapeutic Ultrasound Applications in Craniofacial Growth, Healing and Tissue Engineering. Rejuvenation Res 10(3):**367-371**

El-Bialy T, El-Shamy I, Graber TM (2003a) Growth Modification of the Rabbit Mandible Using Therapeutic Ultrasound: Is It Possible to Enhance Functional Appliance Results? Angle Orthod 73(6):**631-639**

El-Bialy T, El-Shamy I, Graber TM (2004) Repair of Orthodontically Induced Root Resorption by Ultrasound in Humans. Am J Orthod Dentofacial Orthop 126(2):**186-193**

El-Bialy T, Hassan A, Albaghdadi T, Fouad HA, Maimani AR (2006) Growth Modification of the Mandible with Ultrasound in Baboons: A Preliminary Report. Am J Orthod Dentofacial Orthop 130(4):**435 e437-414**

El-Bialy TH, el-Moneim Zaki A, Evans CA (2003b) Effect of Ultrasound on Rabbit Mandibular Incisor Formation and Eruption after Mandibular Osteodistraction. Am J Orthod Dentofacial Orthop 124(4):**427-434**

El-Bialy TH, Elgazzar RF, Megahed EE, Royston TJ (2008) Effects of Ultrasound Modes on Mandibular Osteodistraction. J Dent Res 87(10):**953-957**

El-Bialy TH, Royston TJ, Magin RL, Evans CA, Zaki Ael M, Frizzell LA (2002) The Effect of Pulsed Ultrasound on Mandibular Distraction. Ann Biomed Eng 30(10):**1251-1261**

ElHag M, Coghlan K, Christmas P, Harvey W, Harris M (1985) The Anti-Inflammatory Effects of Dexamethasone and Therapeutic Ultrasound in Oral Surgery. Br J Oral Maxillofac Surg 23(1):**17-23**

Enwemeka CS, Rodriguez O, Mendosa S (1990) The Biomechanical Effects of Low-Intensity Ultrasound on Healing Tendons. Ultrasound Med Biol 16(8):**801-807**

Erickson RI (1964) Ultrasound--a Useful Adjunct in Temporomandibular Joint Therapy. Oral Surg Oral Med Oral Pathol 18:**176-179**

Everbach EC, Francis CW (2000) Cavitational Mechanisms in Ultrasound-Accelerated Thrombolysis at 1 Mhz. Ultrasound Med Biol 26(7):**1153-1160**

Felver B, King DC, Lea SC, Price GJ, Damien Walmsley A (2009) Cavitation Occurrence around Ultrasonic Dental Scalers. Ultrason Sonochem 16(5):**692-697**

Feril LB, Jr., Kondo T (2004) Biological Effects of Low Intensity Ultrasound: The Mechanism Involved, and Its Implications on Therapy and on Biosafety of Ultrasound. J Radiat Res (Tokyo) 45(4):**479-489**

Finkelman RD, Mohan S, Jennings JC, Taylor AK, Jepsen S, Baylink DJ (1990) Quantitation of Growth Factors Igf-I, Sgf/Igf-Ii, and Tgf-Beta in Human Dentin. J Bone Miner Res 5(7):**717-723**

Fischer C, Wennberg A, Fischer RG, Attstrom R (1991) Clinical Evaluation of Pulp and Dentine Sensitivity after Supragingival and Subgingival Scaling. Endod Dent Traumatol 7(6):**259-265**

Flemming K, Cullum N (2000) Therapeutic Ultrasound for Venous Leg Ulcers. Cochrane database of systematic reviews(4):**CD001180**

Flour MP, Ronot X, Vincent F, Benoit B, Adolphe M (1992) Differential Temperature Sensitivity of Cultured Cells from Cartilaginous or Bone Origin. Biol Cell 75(1):**83-87**

Fujita S, Hideshima K, Ikeda T (2006) Nestin Expression in Odontoblasts and Odontogenic Ectomesenchymal Tissue of Odontogenic Tumours. J Clin Pathol 59(3):**240-245**

Fulton AF (1984) The Cytoskeleton: Cellular Architecture and Choreography. Chapman and Hall, London, UK.

Gallo JA, Draper DO, Brody LT, Fellingham GW (2004) A Comparison of Human Muscle Temperature Increases During 3-Mhz Continuous and Pulsed Ultrasound with Equivalent Temporal Average Intensities. J Orthop Sports Phys Ther 34(7):**395-401**

Gankerseer EJ, Walmsley AD (1987) Preliminary Investigation into the Performance of a Sonic Scaler. J Periodontol 58(11):**780-784**

Garcia-Cardena G, Anderson KR, Mauri L, Gimbrone MA, Jr. (2000) Distinct Mechanical Stimuli Differentially Regulate the Pi3k/Akt Survival Pathway in Endothelial Cells. Ann N Y Acad Sci 902:**294-297**

Garrido C, Gurbuxani S, Ravagnan L, Kroemer G (2001) Heat Shock Proteins: Endogenous Modulators of Apoptotic Cell Death. Biochem Biophys Res Commun 286(3):**433-442**

Giancotti FG, Ruoslahti E (1999) Integrin Signaling. Science 285(5430):**1028-1032**

Goldberg M, Smith AJ (2004) Cells and Extracellular Matrices of Dentin and Pulp: A Biological Basis for Repair and Tissue Engineering. Crit Rev Oral Biol Med 15(1):**13-27**

Goldman DE, Lepeschkin WW (1952) Injury to Living Cells in Standing Sound Waves. J Cell Physiol 40(2):**255-268**

Goncalves SB, Dong Z, Bramante CM, Holland GR, Smith AJ, Nor JE (2007) Tooth Slice-Based Models for the Study of Human Dental Pulp Angiogenesis. J Endod 33(7):**811-814**

Gong Y, Slee RB, Fukai N, Rawadi G, Roman-Roman S, Reginato AM, Wang H, Cundy T, Glorieux FH, Lev D, Zacharin M, Oexle K, Marcelino J, Suwairi W, Heeger S, Sabatakos G, Apte S, Adkins WN, Allgrove J, Arslan-Kirchner M, Batch JA, Beighton P, Black GC, Boles RG, Boon LM, Borrone C, Brunner HG, Carle GF, Dallapiccola B, De Paepe A, Floege B, Halfhide ML, Hall B, Hennekam RC, Hirose T, Jans A, Juppner H, Kim CA, Keppler-Noreuil K, Kohlschuetter A, LaCombe D, Lambert M, Lemyre E, Letteboer T, Peltonen L, Ramesar RS, Romanengo M, Somer H, Steichen-Gersdorf E, Steinmann B, Sullivan B, Superti-Furga A, Swoboda W, van den Boogaard MJ, Van Hul W, Vikkula M, Votruba M, Zabel B, Garcia T, Baron R, Olsen BR, Warman ML (2001) Ldl Receptor-Related Protein 5 (Lrp5) Affects Bone Accrual and Eye Development. Cell 107(4):**513-523**

Goss SA, Frizzell LA, Dunn F (1979) Ultrasonic Absorption and Attenuation in Mammalian Tissues. Ultrasound Med Biol 5(2):**181-186**

Gotlieb AI, Spector W (1981) Migration into an in Vitro Experimental Wound: A Comparison of Porcine Aortic Endothelial and Smooth Muscle Cells and the Effect of Culture Irradiation. Am J Pathol 103(2):**271-282**

Grando Mattuella L, Westphalen Bento L, de Figueiredo JA, Nor JE, de Araujo FB, Fossati AC (2007) Vascular Endothelial Growth Factor and Its Relationship with the Dental Pulp. J Endod 33(5):**524-530**

Gregory CA, Gunn WG, Peister A, Prockop DJ (2004) An Alizarin Red-Based Assay of Mineralization by Adherent Cells in Culture: Comparison with Cetylpyridinium Chloride Extraction. Anal Biochem 329(1):**77-84**

Gronthos S, Mankani M, Brahim J, Robey PG, Shi S (2000) Postnatal Human Dental Pulp Stem Cells (Dpscs) in Vitro and in Vivo. Proc Natl Acad Sci U S A 97(25):**13625-13630**

Grossi A, Yadav K, Lawson MA (2007) Mechanical Stimulation Increases Proliferation, Differentiation and Protein Expression in Culture: Stimulation Effects Are Substrate Dependent. J Biomech 40(15):**3354-3362**

Gustin JA, Korgaonkar CK, Pincheira R, Li Q, Donner DB (2006) Akt Regulates Basal and Induced Processing of Nf-Kappab2 (P100) to P52. J Biol Chem 281(24):**16473-16481**

Han SI, Oh SY, Jeon WJ, Kim JM, Lee JH, Chung HY, Choi YH, Yoo MA, Kim HD, Kang HS (2002) Mild Heat Shock Induces Cyclin D1 Synthesis through Multiple Ras Signal Pathways. FEBS Lett 515(1-3):**141-145**

Han SI, Oh SY, Woo SH, Kim KH, Kim JH, Kim HD, Kang HS (2001) Implication of a Small Gtpase Rac1 in the Activation of C-Jun N-Terminal Kinase and Heat Shock Factor in Response to Heat Shock. J Biol Chem 276(3):**1889-1895**

Hanks CT, Fang D, Sun Z, Edwards CA, Butler WT (1998a) Dentin-Specific Proteins in Mdpc-23 Cell Line. Eur J Oral Sci 106 Suppl 1:**260-266**

Hanks CT, Sun ZL, Fang DN, Edwards CA, Wataha JC, Ritchie HH, Butler WT (1998b) Cloned 3t6 Cell Line from Cd-1 Mouse Fetal Molar Dental Papillae. Connect Tissue Res 37(3-4):**233-249**

Harle J, Mayia F, Olsen I, Salih V (2005) Effects of Ultrasound on Transforming Growth Factor-Beta Genes in Bone Cells. Eur Cell Mater 10:**70-76; discussion 76**

Harle J, Salih V, Knowles JC, Mayia F, Olsen I (2001a) Effects of Therapeutic Ultrasound on Osteoblast Gene Expression. J Mater Sci Mater Med 12(10-12):**1001-1004**

Harle J, Salih V, Mayia F, Knowles JC, Olsen I (2001b) Effects of Ultrasound on the Growth and Function of Bone and Periodontal Ligament Cells in Vitro. Ultrasound Med Biol 27(4):**579-586**

Harris M (1992) The Conservative Management of Osteoradionecrosis of the Mandible with Ultrasound Therapy. Br J Oral Maxillofac Surg 30(5):**313-318**

Hashish I, Harvey W, Harris M (1986) Anti-Inflammatory Effects of Ultrasound Therapy: Evidence for a Major Placebo Effect. Br J Rheumatol 25(1):**77-81**

Hatai T, Yokozeki M, Funato N, Baba Y, Moriyama K, Ichijo H, Kuroda T (2001) Apoptosis of Periodontal Ligament Cells Induced by Mechanical Stress During Tooth Movement. Oral Dis 7(5):**287-290**

Hauser J, Hauser M, Muhr G, Esenwein S (2008a) Ultrasound-Induced Modifications of Cytoskeletal Components in Osteoblast-Like Saos-2 Cells. J Orthop Res:**In Press**

Hauser J, Hauser M, Muhr G, Esenwein S (2008b) Ultrasound-Induced Modifications of Cytoskeletal Components in Osteoblast-Like Saos-2 Cells. J Orthop Res

Hayton MJ, Dillon JP, Glynn D, Curran JM, Gallagher JA, Buckley KA (2005) Involvement of Adenosine 5'-Triphosphate in Ultrasound-Induced Fracture Repair. Ultrasound Med Biol 31(8):**1131-1138**

He WX, Niu ZY, Zhao SL, Jin WL, Gao J, Smith AJ (2004) Tgf-Beta Activated Smad Signalling Leads to a Smad3-Mediated Down-Regulation of Dspp in an Odontoblast Cell Line. Arch Oral Biol 49(11):**911-918**

Heckman JD, Ryaby JP, McCabe J, Frey JJ, Kilcoyne RF (1994) Acceleration of Tibial Fracture-Healing by Non-Invasive, Low-Intensity Pulsed Ultrasound. J Bone Joint Surg Am 76(1):**26-34**

Hill GE, Fenwick S, Matthews BJ, Chivers RA, Southgate J (2005) The Effect of Low-Intensity Pulsed Ultrasound on Repair of Epithelial Cell Monolayers in Vitro. Ultrasound Med Biol 31(12):**1701-1706**

Hisaka M, Sugiura T, Kawata S (2001) Optical Cross-Sectional Imaging with Pulse Ultrasound Wave Assistance. J Opt Soc Am A Opt Image Sci Vis 18(7):**1531-1534**

Hiyama A, Mochida J, Iwashina T, Omi H, Watanabe T, Serigano K, Iwabuchi S, Sakai D (2007) Synergistic Effect of Low-Intensity Pulsed Ultrasound on Growth Factor Stimulation of Nucleus Pulposus Cells. J Orthop Res 25(12):**1574-1581**

Hoemann CD, El-Gabalawy H, McKee MD (2009) In Vitro Osteogenesis Assays: Influence of the Primary Cell Source on Alkaline Phosphatase Activity and Mineralization. Pathol Biol (Paris) 57(4):**318-323**

Honda MJ, Shinohara Y, Sumita Y, Tonomura A, Kagami H, Ueda M (2006) Shear Stress Facilitates Tissue-Engineered Odontogenesis. Bone 39(1):**125-133**

Howry DH, Bliss WR (1952) Ultrasonic Visualization of Soft Tissue Structures of the Body. J Lab Clin Med 40(4):**579-592**

Hu CC, Zhang C, Qian Q, Tatum NB (1998) Reparative Dentin Formation in Rat Molars after Direct Pulp Capping with Growth Factors. J Endod 24(11):**744-751**

Huang MH, Yang RC, Ding HJ, Chai CY (1999) Ultrasound Effect on Level of Stress Proteins and Arthritic Histology in Experimental Arthritis. Arch Phys Med Rehabil 80(5):**551-556**

Ikai H, Tamura T, Watanabe T, Itou M, Sugaya A, Iwabuchi S, Mikuni-Takagaki Y, Deguchi S (2008) Low-Intensity Pulsed Ultrasound Accelerates Periodontal Wound Healing after Flap Surgery. J Periodontal Res 43(2):**212-216**

Ingber D (1991) Integrins as Mechanochemical Transducers. Curr Opin Cell Biol 3(5):**841-848**

Ingber DE, Dike L, Hansen L, Karp S, Liley H, Maniotis A, McNamee H, Mooney D, Plopper G, Sims J, et al. (1994) Cellular Tensegrity: Exploring How Mechanical Changes in the Cytoskeleton Regulate Cell Growth, Migration, and Tissue Pattern During Morphogenesis. Int Rev Cytol 150:**173-224**

Iohara K, Nakashima M, Ito M, Ishikawa M, Nakasima A, Akamine A (2004) Dentin Regeneration by Dental Pulp Stem Cell Therapy with Recombinant Human Bone Morphogenetic Protein 2. J Dent Res 83(8):**590-595**

Ivaska J, Pallari H-M, Nevo J, Eriksson JE (2007) Novel Functions of Vimentin in Cell Adhesion, Migration, and Signaling. Experimental Cell Research 313(10):**2050-2062**

Iwai T, Harada Y, Imura K, Iwabuchi S, Murai J, Hiramatsu K, Myoui A, Yoshikawa H, Tsumaki N (2007) Low-Intensity Pulsed Ultrasound Increases Bone Ingrowth into Porous Hydroxyapatite Ceramic. J Bone Miner Metab 25(6):**392-399**

Jacobson L, Blomlof J, Lindskog S (1994) Root Surface Texture after Different Scaling Modalities. Scand J Dent Res 102(3):**156-160**

Kasturi G, Adler RA (2011) Mechanical Means to Improve Bone Strength: Ultrasound and Vibration. Curr Rheumatol Rep 13(3):**251-256**

Kaufman GE, Miller MW (1978) Growth Retardation in Chinese Hamster V-79 Cells Exposed to 1 Mhz Ultrasound. Ultrasound Med Biol 4(2):**139-144**

Kaufman GE, Miller MW, Griffiths TD, Ciaravino V, Carstensen EL (1977) Lysis and Viability of Cultured Mammalian Cells Exposed to 1 Mhz Ultrasound. Ultrasound Med Biol 3(1):**21-25**

Kerr CL, Gregory DW, Shammari M, Watmough DJ, Wheatley DN (1989) Differing Effects of Ultrasound-Irradiation on Suspension and Monolayer Cultured Hela Cells, Investigated by Scanning Electron Microscopy. Ultrasound Med Biol 15(4):**397-401**

Khanna A, Nelmes RT, Gougoulias N, Maffulli N, Gray J (2009) The Effects of Lipus on Soft-Tissue Healing: A Review of Literature. Br Med Bull 89:**169-182**

Kim CH, Cho YS, Chun YS, Park JW, Kim MS (2002) Early Expression of Myocardial Hif-1alpha in Response to Mechanical Stresses: Regulation by Stretch-Activated Channels and the Phosphatidylinositol 3-Kinase Signaling Pathway. Circ Res 90(2):**E25-33**

Klug W, Franke WG, Knoch HG (1986) Scintigraphic Control of Bone-Fracture Healing under Ultrasonic Stimulation: An Animal Experimental Study. Eur J Nucl Med 11(12):**494-497**

Knight KL, O Draper D (2007) Therapeutic Modalities: The Art and Science. Lippincott Williams & Wilkins, UK. **p260-261**

Kobayashi Y, Sakai D, Iwashina T, Iwabuchi S, Mochida J (2009) Low-Intensity Pulsed Ultrasound Stimulates Cell Proliferation, Proteoglycan Synthesis and Expression of Growth Factor-Related Genes in Human Nucleus Pulposus Cell Line. Eur Cell Mater 17:**15-22**

Koempel JA, Patt BS, O'Grady K, Wozney J, Toriumi DM (1998) The Effect of Recombinant Human Bone Morphogenetic Protein-2 on the Integration of Porous Hydroxyapatite Implants with Bone. J Biomed Mater Res 41(3):**359-363**

Kong X, Cao M, Ye R, Ding Y (2010) Orthodontic Force Accelerates Dentine Mineralization During Tooth Development in Juvenile Rats. Tohoku J Exp Med 221(4):**265-270**

Korstjens CM, van der Rijt RH, Albers GH, Semeins CM, Klein-Nulend J (2008) Low-Intensity Pulsed Ultrasound Affects Human Articular Chondrocytes in Vitro. Med Biol Eng Comput 46(12):**1263-1270**

Krifka S, Seidenader C, Hiller KA, Schmalz G, Schweikl H (2011) Oxidative Stress and Cytotoxicity Generated by Dental Composites in Human Pulp Cells. Clin Oral Investig

Krishnan V, Bryant HU, Macdougald OA (2006) Regulation of Bone Mass by Wnt Signaling. J Clin Invest 116(5):**1202-1209**

Kristiansen TK, Ryaby JP, McCabe J, Frey JJ, Roe LR (1997) Accelerated Healing of Distal Radial Fractures with the Use of Specific, Low-Intensity Ultrasound. A Multicenter, Prospective, Randomized, Double-Blind, Placebo-Controlled Study. J Bone Joint Surg Am 79(7):**961-973**

Kruse DE, Mackanos MA, O'Connell-Rodwell CE, Contag CH, Ferrara KW (2008) Short-Duration-Focused Ultrasound Stimulation of Hsp70 Expression in Vivo. Phys Med Biol 53(13):**3641-3660**

Lachgar S, Charveron M, Gall Y, Plouet J, Bonafe JL (1996) Vascular Endothelial Cells: Targets for Studying the Activity of Hair Follicle Cell-Produced Vegf. Cell Biol Toxicol 12(4-6):**331-334**

Landry J, Samson S, Chretien P (1986) Hyperthermia-Induced Cell Death, Thermotolerance, and Heat Shock Proteins in Normal, Respiration-Deficient, and Glycolysis-Deficient Chinese Hamster Cells. Cancer Res 46(1):**324-327**

Lau KH, Kapur S, Kesavan C, Baylink DJ (2006) Up-Regulation of the Wnt, Estrogen Receptor, Insulin-Like Growth Factor-I, and Bone Morphogenetic Protein Pathways in C57bl/6j Osteoblasts as Opposed to C3h/Hej Osteoblasts in Part Contributes to the Differential Anabolic Response to Fluid Shear. J Biol Chem 281(14):**9576-9588**

Lea SC, Felver B, Landini G, Walmsley AD (2009) Ultrasonic Scaler Oscillations and Tooth-Surface Defects. J Dent Res 88(3):**229-234**

Lea SC, Landini G, Walmsley AD (2003) Displacement Amplitude of Ultrasonic Scaler Inserts. J Clin Periodontol 30(6):**505-510**

Lee A, Heasman PA, Kelly PJ (1996) An in Vitro Comparative Study of a Reciprocating Scaler for Root Surface Debridement. J Dent 24(1-2):**81-86**

Lee HJ, Choi BH, Min BH, Park SR (2007) Low-Intensity Ultrasound Inhibits Apoptosis and Enhances Viability of Human Mesenchymal Stem Cells in Three-Dimensional Alginate Culture During Chondrogenic Differentiation. Tissue Eng 13(5):**1049-1057**

Lee HJ, Choi BH, Min BH, Son YS, Park SR (2006) Low-Intensity Ultrasound Stimulation Enhances Chondrogenic Differentiation in Alginate Culture of Mesenchymal Stem Cells. Artif Organs 30(9):**707-715**

Leung KS, Cheung WH, Zhang C, Lee KM, Lo HK (2004) Low Intensity Pulsed Ultrasound Stimulates Osteogenic Activity of Human Periosteal Cells. Clin Orthop Relat Res(418):**253-259**

Li JG, Chang WH, Lin JC, Sun JS (2002) Optimum Intensities of Ultrasound for Pge(2) Secretion and Growth of Osteoblasts. Ultrasound Med Biol 28(5):**683-690**

Liang CC, Park AY, Guan JL (2007) In Vitro Scratch Assay: A Convenient and Inexpensive Method for Analysis of Cell Migration in Vitro. Nat Protoc 2(2):**329-333**

Lianjia Y, Yuhao G, White FH (1993) Bovine Bone Morphogenetic Protein-Induced Dentinogenesis. Clin Orthop Relat Res(295):**305-312**

Lind M, Overgaard S, Song Y, Goodman SB, Bunger C, Soballe K (2000) Osteogenic Protein 1 Device Stimulates Bone Healing to Hydroxyapaptite-Coated and Titanium Implants. J Arthroplasty 15(3):**339-346**

Linde A, Goldberg M (1993) Dentinogenesis. Crit Rev Oral Biol Med 4(5):**679-728**

Loch EG, Fischer AB, Kuwert E (1971) Effect of Diagnostic and Therapeutic Intensities of Ultrasonics on Normal and Malignant Human Cells in Vitro. Am J Obstet Gynecol 110(4):**457-460**

Locke M, Nussbaum E (2001) Continuous and Pulsed Ultrasound Do Not Increase Heat Shock Protein 72 Content. Ultrasound Med Biol 27(10):**1413-1419**

Logan CY, Nusse R (2004) The Wnt Signaling Pathway in Development and Disease. Annu Rev Cell Dev Biol 20:**781-810**

Love LA, Kremkau FW (1980) Intracellular Temperature Distribution Produced by Ultrasound. Journal of the Acoustical Society of America 67:**1045-1050**

Lu H, Qin L, Cheung W, Lee K, Wong W, Leung K (2008a) Low-Intensity Pulsed Ultrasound Accelerated Bone-Tendon Junction Healing through Regulation of Vascular Endothelial Growth Factor Expression and Cartilage Formation. Ultrasound Med Biol 34(8):**1248-1260**

Lu HB, Xu DQ, Wang XY, Hu JZ, Duan CY, Zhang BL (2008b) [Effect of Low-Intensity Pulsed Ultrasound on the Enchondral Bone Formation in Posterolateral Lumbar Fusion in Rabbits]. Zhong Nan Da Xue Xue Bao Yi Xue Ban 33(8):**688-692**

Lu Y, Ye L, Yu S, Zhang S, Xie Y, McKee MD, Li YC, Kong J, Eick JD, Dallas SL, Feng JQ (2007) Rescue of Odontogenesis in Dmp1-Deficient Mice by Targeted Re-Expression of Dmp1 Reveals Roles for Dmp1 in Early Odontogenesis and Dentin Apposition in Vivo. Dev Biol 303(1):**191-201**

Luo T, Zhang W, Shi B, Cheng X, Zhang Y (2011) Enhanced Bone Regeneration around Dental Implant with Bone Morphogenetic Protein 2 Gene and Vascular Endothelial Growth Factor Protein Delivery. Clin Oral Implants Res

Lynch SE, Buser D, Hernandez RA, Weber HP, Stich H, Fox CH, Williams RC (1991) Effects of the Platelet-Derived Growth Factor/Insulin-Like Growth Factor-I Combination on Bone Regeneration around Titanium Dental Implants. Results of a Pilot Study in Beagle Dogs. J Periodontol 62(11):**710-716**

Maeda K, Murao F (1977) Studies on the Influence of Ultrasound Irritation of the Growth of Cultured Cell in Vitro. In: White D, Brown RE (eds) Ultrasonics in Medicine. Plenum Press, **p2045**

Martinez de Albornoz P, Khanna A, Longo UG, Forriol F, Maffulli N (2011) The Evidence of Low-Intensity Pulsed Ultrasound for in Vitro, Animal and Human Fracture Healing. Br Med Bull

Marvel S, Okrasinski S, Bernacki SH, Loboa E, Dayton PA (2010) The Development and Validation of a Lipus System with Preliminary Observations of Ultrasonic Effects on Human Adult Stem Cells. IEEE Trans Ultrason Ferroelectr Freq Control 57(9):**1977-1984**

Mathieu S, El-Battari A, Dejou J, About I (2005) Role of Injured Endothelial Cells in the Recruitment of Human Pulp Cells. Archives of Oral Biology 50(2):**109**

Matsushita K, Motani R, Sakuta T, Yamaguchi N, Koga T, Matsuo K, Nagaoka S, Abeyama K, Maruyama I, Torii M (2000) The Role of Vascular Endothelial Growth Factor in Human Dental Pulp Cells: Induction of Chemotaxis, Proliferation, and Differentiation and Activation of the Ap-1-Dependent Signaling Pathway. J Dent Res 79(8):**1596-1603**

McCormick SM, Saini V, Yazicioglu Y, Demou ZN, Royston TJ (2006) Interdependence of Pulsed Ultrasound and Shear Stress Effects on Cell Morphology and Gene Expression. Ann Biomed Eng 34(3):**436-445**

McLachlan JL, Smith AJ, Cooper PR (2003) Piezo-Power Microdissection of Mature Human Dental Tissue. Arch Oral Biol 48(10):**731-736**

Meazzini MC, Toma CD, Schaffer JL, Gray ML, Gerstenfeld LC (1998) Osteoblast Cytoskeletal Modulation in Response to Mechanical Strain in Vitro. J Orthop Res 16(2):**170-180**

Meyer U (2009) Fundamentals of Tissue Engineering and Regenerative Medicine. Springer, Berlin ; [London]. **pxxvi, 1049 p.**

Miller DL, Thomas RM (1995) Ultrasound Contrast Agents Nucleate Inertial Cavitation in Vitro. Ultrasound Med Biol 21(8):**1059-1065**

Miller MW, Miller DL, Brayman AA (1996) A Review of in Vitro Bioeffects of Inertial Ultrasonic Cavitation from a Mechanistic Perspective. Ultrasound Med Biol 22(9):**1131-1154**

Miller MW, Ziskin MC (1989) Biological Consequences of Hyperthermia. Ultrasound Med Biol 15(8):**707-722**

Mitragotri S (2005) Healing Sound: The Use of Ultrasound in Drug Delivery and Other Therapeutic Applications. Nat Rev Drug Discov 4(3):**255-260**

Mitragotri S, Blankschtein D, Langer R (1996) Transdermal Drug Delivery Using Low-Frequency Sonophoresis. Pharm Res 13(3):**411-420**

Mitsiadis TA, Rahiotis C (2004) Parallels between Tooth Development and Repair: Conserved Molecular Mechanisms Following Carious and Dental Injury. J Dent Res 83(12):**896-902**

Mizrahi N, Seliktar D, Kimmel E (2007) Ultrasound-Induced Angiogenic Response in Endothelial Cells. Ultrasound Med Biol 33(11):**1818-1829**

Mooney DJ, Powell C, Piana J, Rutherford B (1996) Engineering Dental Pulp-Like Tissue in Vitro. Biotechnol Prog 12(6):**865-868**

Mortimer AJ, Dyson M (1988) The Effect of Therapeutic Ultrasound on Calcium Uptake in Fibroblasts. Ultrasound Med Biol 14(6):**499-506**

Moss-Salentijn L, Hendricks-Klyvert M (1990) Dental and Oral Tissues; an Introduction, 3rd edn. Lea and Febiger, Philadelphia. **p204, 211, 216, 241**

Mostafa NZ, Uludag H, Dederich DN, Doschak MR, El-Bialy TH (2009) Anabolic Effects of Low-Intensity Pulsed Ultrasound on Human Gingival Fibroblasts. Arch Oral Biol 54(8):**743-748**

Mukai S, Ito H, Nakagawa Y, Akiyama H, Miyamoto M, Nakamura T (2005) Transforming Growth Factor-Beta1 Mediates the Effects of Low-Intensity Pulsed Ultrasound in Chondrocytes. Ultrasound Med Biol 31(12):**1713-1721**

Murphy WL, Peters MC, Kohn DH, Mooney DJ (2000) Sustained Release of Vascular Endothelial Growth Factor from Mineralized Poly(Lactide-Co-Glycolide) Scaffolds for Tissue Engineering. Biomaterials 21(24):**2521-2527**

Murray PE, About I, Franquin JC, Remusat M, Smith AJ (2001) Restorative Pulpal and Repair Responses. J Am Dent Assoc 132(4):**482-491**

Murray PE, Lumley PJ, Ross HF, Smith AJ (2000) Tooth Slice Organ Culture for Cytotoxicity Assessment of Dental Materials. Biomaterials 21(16):**1711-1721**

Nakashima M (1994) Induction of Dentin Formation on Canine Amputated Pulp by Recombinant Human Bone Morphogenetic Proteins (Bmp)-2 and -4. J Dent Res 73(9):**1515-1522**

Nakashima M (2005) Bone Morphogenetic Proteins in Dentin Regeneration for Potential Use in Endodontic Therapy. Cytokine Growth Factor Rev 16(3):**369-376**

Nakashima M, Iohara K, Ishikawa M, Ito M, Tomokiyo A, Tanaka T, Akamine A (2004) Stimulation of Reparative Dentin Formation by Ex Vivo Gene Therapy Using Dental Pulp Stem Cells Electrotransfected with Growth/Differentiation Factor 11 (Gdf11). Hum Gene Ther 15(11):**1045-1053**

Nakashima M, Tachibana K, Iohara K, Ito M, Ishikawa M, Akamine A (2003) Induction of Reparative Dentin Formation by Ultrasound-Mediated Gene Delivery of Growth/Differentiation Factor 11. Hum Gene Ther 14(6):**591-597**

Nanci A (2003) Enamel: Composition, Formation, and Structure. In: A N (ed) Ten Cate's Oral Histology: Development, Structure and Function. Mosby Incorporation, **p145, 194, 195 213, 218**

Narayanan K, Srinivas R, Ramachandran A, Hao J, Quinn B, George A (2001) Differentiation of Embryonic Mesenchymal Cells to Odontoblast-Like Cells by Overexpression of Dentin Matrix Protein 1. Proc Natl Acad Sci U S A 98(8):**4516-4521**

Naruse K, Miyauchi A, Itoman M, Mikuni-Takagaki Y (2003) Distinct Anabolic Response of Osteoblast to Low-Intensity Pulsed Ultrasound. Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research 18(2):**360-369**

Nicoll BK, Peters RJ (1998) Heat Generation During Ultrasonic Instrumentation of Dentin as Affected by Different Irrigation Methods. J Periodontol 69(8):**884-888**

Nishimura M, Chiba M, Ohashi T, Sato M, Shimizu Y, Igarashi K, Mitani H (2008) Periodontal Tissue Activation by Vibration: Intermittent Stimulation by Resonance Vibration Accelerates Experimental Tooth Movement in Rats. Am J Orthod Dentofacial Orthop 133(4):**572-583**

Nomi M, Miyake H, Sugita Y, Fujisawa M, Soker S (2006) Role of Growth Factors and Endothelial Cells in Therapeutic Angiogenesis and Tissue Engineering. Curr Stem Cell Res Ther 1(3):**333-343**

Nussbaum EL, Locke M (2007a) Heat Shock Protein Expression in Rat Skeletal Muscle after Repeated Applications of Pulsed and Continuous Ultrasound. Arch Phys Med Rehabil 88(6):**785-790**

Nussbaum EL, Locke M (2007b) Heat Shock Protein Expression in Rat Skeletal Muscle after Repeated Applications of Pulsed and Continuous Ultrasound. Archives of Physical Medicine and Rehabilitation 88(6):**785-790**

Nyborg H, Brannstrom M (1968) Pulp Reaction to Heat. J Prosthet Dent 19(6):**605-612**

Nyborg WL (1987) Determining Risk to Subjects: Exposure to Ultrasound. Irb 9(3):**1-5**

O'Reilly MA, Huang Y, Hynynen K (2010) The Impact of Standing Wave Effects on Transcranial Focused Ultrasound Disruption of the Blood-Brain Barrier in a Rat Model. Phys Med Biol 55(18):**5251-5267**

Ohshima H (1990) Ultrastructural Changes in Odontoblasts and Pulp Capillaries Following Cavity Preparation in Rat Molars. Arch Histol Cytol 53(4):**423-438**

Olkku A, Leskinen JJ, Lammi MJ, Hynynen K, Mahonen A (2010) Ultrasound-Induced Activation of Wnt Signaling in Human Mg-63 Osteoblastic Cells. Bone 47(2):**320-330**

Olsson AK, Dimberg A, Kreuger J, Claesson-Welsh L (2006) Vegf Receptor Signalling - in Control of Vascular Function. Nat Rev Mol Cell Biol 7(5):**359-371**

Onyekwelu O, Seppala M, Zoupa M, Cobourne MT (2007) Tooth Development: 2. Regenerating Teeth in the Laboratory. Dent Update 34(1):**20-22, 25-26, 29**

Orlandini M, Semboloni S, Oliviero S (2003) Beta-Catenin Inversely Regulates Vascular Endothelial Growth Factor-D Mrna Stability. J Biol Chem 278(45):**44650-44656**

Orsini G, Ruggeri A, Mazzoni A, Nato F, Falconi M, Putignano A, Di Lenarda R, Nanci A, Breschi L (2008) Immunohistochemical Localization of Dentin Matrix Protein 1 in Human Dentin. Eur J Histochem 52(4):**215-220**

Paliwal S, Mitragotri S (2006) Ultrasound-Induced Cavitation: Applications in Drug and Gene Delivery. Expert Opinion on Drug Delivery 3(6):**713-726**

Paliwal S, Mitragotri S (2008) Therapeutic Opportunities in Biological Responses of Ultrasound. Ultrasonics 48(4):**271-278**

Pallari H-M, Eriksson JE (2006) Intermediate Filaments as Signaling Platforms. Sci. STKE 2006(366):**pe53-**

Papakonstanti EA, Stournaras C (2008) Cell Responses Regulated by Early Reorganization of Actin Cytoskeleton. FEBS Letters 582(14):**2120-2127**

Park HG, Han SI, Oh SY, Kang HS (2005) Cellular Responses to Mild Heat Stress. Cell Mol Life Sci 62(1):**10-23**

Parvizi J, Wu CC, Lewallen DG, Greenleaf JF, Bolander ME (1999) Low-Intensity Ultrasound Stimulates Proteoglycan Synthesis in Rat Chondrocytes by Increasing Aggrecan Gene Expression. J Orthop Res 17(4):**488-494**

Peters MC, Polverini PJ, Mooney DJ (2002) Engineering Vascular Networks in Porous Polymer Matrices. J Biomed Mater Res 60(4):**668-678**

Pilla AA, Mont MA, Nasser PR, Khan SA, Figueiredo M, Kaufman JJ, Siffert RS (1990) Non-Invasive Low-Intensity Pulsed Ultrasound Accelerates Bone Healing in the Rabbit. J Orthop Trauma 4(3):**246-253**

Pinco KA, He W, Yang JT (2002) Alpha4beta1 Integrin Regulates Lamellipodia Protrusion Via a Focal Complex/Focal Adhesion-Independent Mechanism. Mol Biol Cell 13(9):**3203-3217**

Pitt WG, Ross SA (2003) Ultrasound Increases the Rate of Bacterial Cell Growth. Biotechnol Prog 19(3):**1038-1044**

Pollard TD (2003) The Cytoskeleton, Cellular Motility and the Reductionist Agenda. Nature 422(6933):**741-745**

Ponchio L, Duma L, Oliviero B, Gibelli N, Pedrazzoli P, Robustelli della Cuna G (2000) Mitomycin C as an Alternative to Irradiation to Inhibit the Feeder Layer Growth in Long-Term Culture Assays. Cytotherapy 2(4):**281-286**

Qin C, Baba O, Butler WT (2004) Post-Translational Modifications of Sibling Proteins and Their Roles in Osteogenesis and Dentinogenesis. Crit Rev Oral Biol Med 15(3):**126-136**

Quarles LD, Yohay DA, Lever LW, Caton R, Wenstrup RJ (1992) Distinct Proliferative and Differentiated Stages of Murine Mc3t3-E1 Cells in Culture: An in Vitro Model of Osteoblast Development. J Bone Miner Res 7(6):**683-692**

Ramirez A, Schwane JA, McFarland C, Starcher B (1997) The Effect of Ultrasound on Collagen Synthesis and Fibroblast Proliferation in Vitro. Med Sci Sports Exerc 29(3):**326-332**

Ramli R, Reher P, Harris M, Meghji S (2009) The Effect of Ultrasound on Angiogenesis: An in Vivo Study Using the Chick Chorioallantoic Membrane. Int J Oral Maxillofac Implants 24(4):**591-596**

Raz D, Zaretsky U, Einav S, Elad D (2005) Cellular Alterations in Cultured Endothelial Cells Exposed to Therapeutic Ultrasound Irradiation. Endothelium 12(4):**201-213**

Rego EB, Inubushi T, Kawazoe A, Tanimoto K, Miyauchi M, Tanaka E, Takata T, Tanne K (2010) Ultrasound Stimulation Induces Pge(2) Synthesis Promoting Cementoblastic Differentiation through Ep2/Ep4 Receptor Pathway. Ultrasound Med Biol 36(6):**907-915**

Reher P (1997) Ultrasound for the Treatment of Osteoradionecrosis. J Oral Maxillofac Surg 55(10):**1193-1194**

Reher P, Doan N, Bradnock B, Meghji S, Harris M (1998) Therapeutic Ultrasound for Osteoradionecrosis: An in Vitro Comparison between 1 Mhz and 45 Khz Machines. Eur J Cancer 34(12):**1962-1968**

Reher P, Doan N, Bradnock B, Meghji S, Harris M (1999) Effect of Ultrasound on the Production of Il-8, Basic Fgf and Vegf. Cytokine 11(6):**416-423**

Reher P, Elbeshir el NI, Harvey W, Meghji S, Harris M (1997) The Stimulation of Bone Formation in Vitro by Therapeutic Ultrasound. Ultrasound Med Biol 23(8):**1251-1258**

Reher P, Harris M, Whiteman M, Hai HK, Meghji S (2002) Ultrasound Stimulates Nitric Oxide and Prostaglandin E2 Production by Human Osteoblasts. Bone 31(1):**236-241**

Rehn AP, Cerny R, Sugars RV, Kaukua N, Wendel M (2008) Osteoadherin Is Upregulated by Mature Osteoblasts and Enhances Their in Vitro Differentiation and Mineralization. Calcif Tissue Int 82(6):**454-464**

Ripamonti U, Reddi AH (1997) Tissue Engineering, Morphogenesis, and Regeneration of the Periodontal Tissues by Bone Morphogenetic Proteins. Crit Rev Oral Biol Med 8(2):**154-163**

Robbins M, McKinney M (1992) Transcriptional Regulation of Neuromodulin (Gap-43) in Mouse Neuroblastoma Clone N1e-115 as Evaluated by the Rt/Pcr Method. Brain Res Mol Brain Res 13(1-2):**83-92**

Roberts-Clark DJ, Smith AJ (2000a) Angiogenic Growth Factors in Human Dentine Matrix. Archives of Oral Biology 45(11):**1013**

Roberts-Clark DJ, Smith AJ (2000b) Angiogenic Growth Factors in Human Dentine Matrix. Arch Oral Biol 45(11):**1013-1016**

Roberts WE, Goodwin WC, Jr., Heiner SR (1981) Cellular Response to Orthodontic Force. Dent Clin North Am 25(1):**3-17**

Robertson VJ, Baker KG (2001) A Review of Therapeutic Ultrasound: Effectiveness Studies. Phys Ther 81(7):**1339-1350**

Robertson VJ, Ward AR (1997) Longwave Ultrasound Reviewed and Reconsidered. Physiotherapy 83(3):**123**

Robinson JA, Chatterjee-Kishore M, Yaworsky PJ, Cullen DM, Zhao W, Li C, Kharode Y, Sauter L, Babij P, Brown EL, Hill AA, Akhter MP, Johnson ML, Recker RR, Komm BS, Bex FJ (2006) Wnt/Beta-Catenin Signaling Is a Normal Physiological Response to Mechanical Loading in Bone. J Biol Chem 281(42):**31720-31728**

Roovers K, Assoian RK (2003) Effects of Rho Kinase and Actin Stress Fibers on Sustained Extracellular Signal-Regulated Kinase Activity and Activation of G(1) Phase Cyclin-Dependent Kinases. Mol Cell Biol 23(12):**4283-4294**

Rubin C, Bolander M, Ryaby JP, Hadjiargyrou M (2001) The Use of Low-Intensity Ultrasound to Accelerate the Healing of Fractures. J Bone Joint Surg Am 83-A(2):**259-270**

Rutherford RB, Wahle J, Tucker M, Rueger D, Charette M (1993) Induction of Reparative Dentine Formation in Monkeys by Recombinant Human Osteogenic Protein-1. Arch Oral Biol 38(7):**571-576**

Sachs F (1991) Mechanical Transduction by Membrane Ion Channels: A Mini Review. Mol Cell Biochem 104(1-2):**57-60**

Sarkar L, Sharpe PT (1999) Expression of Wnt Signalling Pathway Genes During Tooth Development. Mech Dev 85(1-2):**197-200**

Sawakami K, Robling AG, Ai M, Pitner ND, Liu D, Warden SJ, Li J, Maye P, Rowe DW, Duncan RL, Warman ML, Turner CH (2006) The Wnt Co-Receptor Lrp5 Is

Essential for Skeletal Mechanotransduction but Not for the Anabolic Bone Response to Parathyroid Hormone Treatment. J Biol Chem 281(33):**23698-23711**

Saxon LK, Robling AG, Alam I, Turner CH (2005) Mechanosensitivity of the Rat Skeleton Decreases after a Long Period of Loading, but Is Improved with Time Off. Bone 36(3):**454-464**

Scheller EL, Chang J, Wang CY (2008) Wnt/Beta-Catenin Inhibits Dental Pulp Stem Cell Differentiation. J Dent Res 87(2):**126-130**

Scheven BA, Man J, Millard JL, Cooper PR, Lea SC, Walmsley AD, Smith AJ (2009a) Vegf and Odontoblast-Like Cells: Stimulation by Low Frequency Ultrasound. Arch Oral Biol 54(2):**185-191**

Scheven BA, Millard JL, Cooper PR, Lea SC, Walmsley AD, Smith AJ (2007) Short-Term in Vitro Effects of Low Frequency Ultrasound on Odontoblast-Like Cells. Ultrasound in Medicine & Biology 33(9):**1475-1482**

Scheven BA, Shelton RM, Cooper PR, Walmsley AD, Smith AJ (2009b) Therapeutic Ultrasound for Dental Tissue Repair. Med Hypotheses 73(4):**591-593**

Schortinghuis J, Stegenga B, Raghoebar GM, de Bont LGM (2003) Ultrasound Stimulationof Maxillofacial Bone Healing. Crit Rev Oral Biol Med 14(1):**63-74**

Schriefer JL, Warden SJ, Saxon LK, Robling AG, Turner CH (2005) Cellular Accommodation and the Response of Bone to Mechanical Loading. J Biomech 38(9):**1838-1845**

Sena K, Leven RM, Mazhar K, Sumner DR, Virdi AS (2005) Early Gene Response to Low-Intensity Pulsed Ultrasound in Rat Osteoblastic Cells. Ultrasound Med Biol 31(5):**703-708**

Shah S, Walmsley AD, Chapple IL, Lumley PJ (1994) Variability of Sonic Scaling Tip Movement. J Clin Periodontol 21(10):**705-709**

Shaw A, Hodnett M (2008) Calibration and Measurement Issues for Therapeutic Ultrasound. Ultrasonics 48(4):**234-252**

Shiraishi R, Masaki C, Toshinaga A, Okinaga T, Nishihara T, Yamanaka N, Nakamoto T, Hosokawa R (2011) The Effects of Low-Intensity Pulsed Ultrasound Exposure on Gingival Cells. J Periodontol

Sholley MM, Gimbrone MA, Jr., Cotran RS (1977) Cellular Migration and Replication in Endothelial Regeneration: A Study Using Irradiated Endothelial Cultures. Lab Invest 36(1):**18-25**

Simon S, Smith AJ, Berdal A, Lumley PJ, Cooper PR (2010) The Map Kinase Pathway Is Involved in Odontoblast Stimulation Via P38 Phosphorylation. J Endod 36(2):**256-259**

Sloan AJ, Shelton RM, Hann AC, Moxham BJ, Smith AJ (1998) An in Vitro Approach for the Study of Dentinogenesis by Organ Culture of the Dentine-Pulp Complex from Rat Incisor Teeth. Arch Oral Biol 43(6):**421-430**

Sloan AJ, Smith AJ (1999) Stimulation of the Dentine-Pulp Complex of Rat Incisor Teeth by Transforming Growth Factor-Beta Isoforms 1-3 in Vitro. Arch Oral Biol 44(2):**149-156**

Smith AJ (2002) Pulpal Responses to Caries and Dental Repair. Caries Res 36(4):**223-232**

Smith AJ (2003) Vitality of the Dentin-Pulp Complex in Health and Disease: Growth Factors as Key Mediators. J Dent Educ 67(6):**678-689**

Smith AJ (2004) Tooth Tissue Engineering and Regeneration--a Translational Vision! J Dent Res 83(7):**517**

Smith AJ, Cassidy N, Perry H, Begue-Kirn C, Ruch JV, Lesot H (1995a) Reactionary Dentinogenesis. Int J Dev Biol 39(1):**273-280**

Smith AJ, Lumley PJ, Tomson PL, Cooper PR (2008) Dental Regeneration and Materials: A Partnership. Clin Oral Investig 12(2):**103-108**

Smith AJ, Tobias RS, Cassidy N, Begue-Kirn C, Ruch JV, Lesot H (1995b) Influence of Substrate Nature and Immobilization of Implanted Dentin Matrix Components During Induction of Reparative Dentinogenesis. Connect Tissue Res 32(1-4):**291-296**

Smith AJ, Tobias RS, Cassidy N, Plant CG, Browne RM, Begue-Kirn C, Ruch JV, Lesot H (1994) Odontoblast Stimulation in Ferrets by Dentine Matrix Components. Arch Oral Biol 39(1):**13-22**

Smith AJ, Tobias RS, Plant CG, Browne RM, Lesot H, Ruch JV (1990) Preliminary Studies on the in Vivo Morphogenetic Properties of Dentine Matrix Proteins. Biomaterials 11:**22-24**

Soden RI, Botero TM, Hanks CT, Nor JE (2009) Angiogenic Signaling Triggered by Cariogenic Bacteria in Pulp Cells. J Dent Res 88(9):**835-840**

Spinas E (2004) Longevity of Composite Restorations of Traumatically Injured Teeth. Am J Dent 17(6):**407-411**

Srbely JZ (2008) Ultrasound in the Management of Osteoarthritis: Part I: A Review of the Current Literature. J Can Chiropr Assoc 52(1):**30-37**

Stanisic MM, Provo BJ, Larson DL, Kloth LC (2005) Wound Debridement with 25 Khz Ultrasound. Adv Skin Wound Care 18(9):**484-490**

Stein GS, Lian JB (1993) Molecular Mechanisms Mediating Proliferation/Differentiation Interrelationships During Progressive Development of the Osteoblast Phenotype. Endocr Rev 14(4):**424-442**

Stewart HF, Stratmeyer ME (1982) An Overview of Ultrasound: Theory, Measurement, Medical Applications and Biological Effects. Health and Human

Services, HHS Publication (FDA) 82-8190, U.S. Government Printing Office, Washington D.C.

Sudo H, Kodama HA, Amagai Y, Yamamoto S, Kasai S (1983) In Vitro Differentiation and Calcification in a New Clonal Osteogenic Cell Line Derived from Newborn Mouse Calvaria. J Cell Biol 96(1):**191-198**

Sumner DR, Turner TM, Purchio AF, Gombotz WR, Urban RM, Galante JO (1995) Enhancement of Bone Ingrowth by Transforming Growth Factor-Beta. J Bone Joint Surg Am 77(8):**1135-1147**

Sun JS, Hong RC, Chang WH, Chen LT, Lin FH, Liu HC (2001) In Vitro Effects of Low-Intensity Ultrasound Stimulation on the Bone Cells. J Biomed Mater Res 57(3):**449-456**

Sun ZL, Fang DN, Wu XY, Ritchie HH, Begue-Kirn C, Wataha JC, Hanks CT, Butler WT (1998) Expression of Dentin Sialoprotein (Dsp) and Other Molecular Determinants by a New Cell Line from Dental Papillae, Mdpc-23. Connect Tissue Res 37(3-4):**251-261**

Sura H, Shelton RM, Walmsley AD (2001) Osteoblast Viability and Detachment Following Exposure to Ultrasound in Vitro. J Mater Sci Mater Med 12(10-12):**997-1000**

Suzuki A, Takayama T, Suzuki N, Kojima T, Ota N, Asano S, Ito K (2009a) Daily Low-Intensity Pulsed Ultrasound Stimulates Production of Bone Morphogenetic Protein in Ros 17/2.8 Cells. J Oral Sci 51(1):**29-36**

Suzuki A, Takayama T, Suzuki N, Sato M, Fukuda T, Ito K (2009b) Daily Low-Intensity Pulsed Ultrasound-Mediated Osteogenic Differentiation in Rat Osteoblasts. Acta Biochim Biophys Sin (Shanghai) 41(2):**108-115**

Takashi Yamashiro LZ, Yuko Shitaku, Masahiro Saito, Takanori Tsubakimoto, Kenji Takada, Teruko Takano-Yamamoto, Irma Thesleff, (2007) Wnt10a Regulates Dentin

Sialophosphoprotein Mrna Expression and Possibly Links Odontoblast Differentiation and Tooth Morphogenesis. Differentiation 75(5):**452-462**

Takayama T, Suzuki N, Ikeda K, Shimada T, Suzuki A, Maeno M, Otsuka K, Ito K (2007) Low-Intensity Pulsed Ultrasound Stimulates Osteogenic Differentiation in Ros 17/2.8 Cells. Life Sci 80(10):**965-971**

Takeuchi R, Ryo A, Komitsu N, Mikuni-Takagaki Y, Fukui A, Takagi Y, Shiraishi T, Morishita S, Yamazaki Y, Kumagai K, Aoki I, Saito T (2008) Low-Intensity Pulsed Ultrasound Activates the Phosphatidylinositol 3 Kinase/Akt Pathway and Stimulates the Growth of Chondrocytes in Three-Dimensional Cultures: A Basic Science Study. Arthritis Res Ther 10(4):**R77**

Takeuchi R, Saito T, Ishikawa H, Takigami H, Dezawa M, Ide C, Itokazu Y, Ikeda M, Shiraishi T, Morishita S (2006) Effects of Vibration and Hyaluronic Acid on Activation of Three-Dimensional Cultured Chondrocytes. Arthritis Rheum 54(6):**1897-1905**

Tang CH, Yang RS, Huang TH, Lu DY, Chuang WJ, Huang TF, Fu WM (2006) Ultrasound Stimulates Cyclooxygenase-2 Expression and Increases Bone Formation through Integrin, Focal Adhesion Kinase, Phosphatidylinositol 3-Kinase, and Akt Pathway in Osteoblasts. Mol Pharmacol 69(6):**2047-2057**

Tanzer M, Kantor S, Bobyn JD (2001) Enhancement of Bone Growth into Porous Intramedullary Implants Using Non-Invasive Low Intensity Ultrasound. J Orthop Res 19(2):**195-199**

Tecles O, Laurent P, Zygouritsas S, Burger AS, Camps J, Dejou J, About I (2005) Activation of Human Dental Pulp Progenitor/Stem Cells in Response to Odontoblast Injury. Arch Oral Biol 50(2):**103-108**

Telles PD, Hanks CT, Machado MA, Nor JE (2003) Lipoteichoic Acid up-Regulates Vegf Expression in Macrophages and Pulp Cells. J Dent Res 82(6):**466-470**

ter Haar G (1978) Basic Physics of Therapeutic Ultrasound. Physiotherapy 64(4):**100-103**

ter Haar G (1999) Therapeutic Ultrasound. European journal of ultrasound : official journal of the European Federation of Societies for Ultrasound in Medicine and Biology 9(1):**3-9**

ter Haar G, Rivens I, Chen L, Riddler S (1991) High Intensity Focused Ultrasound for the Treatment of Rat Tumours. Phys Med Biol 36(11):**1495-1501**

Terling C, Rass A, Mitsiadis TA, Fried K, Lendahl U, Wroblewski J (1995) Expression of the Intermediate Filament Nestin During Rodent Tooth Development. Int J Dev Biol 39(6):**947-956**

Tezel A, Paliwal S, Shen Z, Mitragotri S (2005) Low-Frequency Ultrasound as a Transcutaneous Immunization Adjuvant. Vaccine 23(29):**3800-3807**

Thesleff I, Sharpe P (1997) Signalling Networks Regulating Dental Development. Mech Dev 67(2):**111-123**

Thi MM, Iacobas DA, Iacobas S, Spray DC (2007) Fluid Shear Stress Upregulates Vascular Endothelial Growth Factor Gene Expression in Osteoblasts. Ann N Y Acad Sci 1117:**73-81**

Thilo B, Chapuis B, Pernet D, Baehni P (1990) [Ultrasonic and Sonic Scalers in Periodontics. A Review]. Schweiz Monatsschr Zahnmed 100(3):**269-273**

Toivola DM, Tao G-Z, Habtezion A, Liao J, Omary MB (2005) Cellular Integrity Plus: Organelle-Related and Protein-Targeting Functions of Intermediate Filaments. Trends in Cell Biology 15(11):**608-617**

Toma CD, Ashkar S, Gray ML, Schaffer JL, Gerstenfeld LC (1997) Signal Transduction of Mechanical Stimuli Is Dependent on Microfilament Integrity:

Identification of Osteopontin as a Mechanically Induced Gene in Osteoblasts. J Bone Miner Res 12(10):**1626-1636**

Tran TA, Roger S, Le Guennec JY, Tranquart F, Bouakaz A (2007) Effect of Ultrasound-Activated Microbubbles on the Cell Electrophysiological Properties. Ultrasound Med Biol 33(1):**158-163**

Trenter SC, Walmsley AD (2003) Ultrasonic Dental Scaler: Associated Hazards. J Clin Periodontol 30(2):**95-101**

Tsai CL, Chang WH, Liu TK (1992) Preliminary Studies of Duration and Intensity of Ultrasonic Treatments on Fracture Repair. Chin J Physiol 35(1):**21-26**

Tsukamoto Y, Fukutani S, Shin-Ike T, Kubota T, Sato S, Suzuki Y, Mori M (1992) Mineralized Nodule Formation by Cultures of Human Dental Pulp-Derived Fibroblasts. Arch Oral Biol 37(12):**1045-1055**

Turner CH (1998) Three Rules for Bone Adaptation to Mechanical Stimuli. Bone 23(5):**399-407**

Tziafas D, Alvanou A, Panagiotakopoulos N, Smith AJ, Lesot H, Komnenou A, Ruch JV (1995) Induction of Odontoblast-Like Cell Differentiation in Dog Dental Pulps after in Vivo Implantation of Dentine Matrix Components. Arch Oral Biol 40(10):**883-893**

Uhlemann C, Heinig B, Wollina U (2003) Therapeutic Ultrasound in Lower Extremity Wound Management. Int J Low Extrem Wounds 2(3):**152-157**

Unsworth J, Kaneez S, Harris S, Ridgway J, Fenwick S, Chenery D, Harrison A (2007) Pulsed Low Intensity Ultrasound Enhances Mineralisation in Preosteoblast Cells. Ultrasound Med Biol 33(9):**1468-1474**

Wagner AC, Weber H, Jonas L, Nizze H, Strowski M, Fiedler F, Printz H, Steffen H, Goke B (1996) Hyperthermia Induces Heat Shock Protein Expression and Protection against Cerulein-Induced Pancreatitis in Rats. Gastroenterology 111(5):**1333-1342**

Waldman SD, Spiteri CG, Grynpas MD, Pilliar RM, Kandel RA (2003) Long-Term Intermittent Shear Deformation Improves the Quality of Cartilaginous Tissue Formed in Vitro. J Orthop Res 21(4):**590-596**

Wang D, Christensen K, Chawla K, Xiao G, Krebsbach PH, Franceschi RT (1999) Isolation and Characterization of Mc3t3-E1 Preosteoblast Subclones with Distinct in Vitro and in Vivo Differentiation/Mineralization Potential. J Bone Miner Res 14(6):**893-903**

Wang SJ, Lewallen DG, Bolander ME, Chao EY, Ilstrup DM, Greenleaf JF (1994) Low Intensity Ultrasound Treatment Increases Strength in a Rat Femoral Fracture Model. J Orthop Res 12(1):**40-47**

Ward AR, Robertson VJ (1996) Comparison of Heating of Nonliving Soft Tissue Produced by 45 Khz and 1 Mhz Frequency Ultrasound Machines. J Orthop Sports Phys Ther 23(4):**258-266**

Warren CG, Koblanski JN, Sigelmann RA (1976) Ultrasound Coupling Media: Their Relative Transmissivity. Arch Phys Med Rehabil 57(5):**218-222**

Warters RL, Roti Roti JL (1982) Hyperthermia and the Cell Nucleus. Radiat Res 92(3):**458-462**

Watson T (2000) The Role of Electrotherapy in Contemporary Physiotherapy Practice. Man Ther 5(3):**132-141**

Webster DF, Harvey W, Dyson M, Pond JB (1980) The Role of Ultrasound-Induced Cavitation in the 'in Vitro' Stimulation of Collagen Synthesis in Human Fibroblasts. Ultrasonics 18(1):**33-37**

Webster DF, Pond JB, Dyson M, Harvey W (1978) The Role of Cavitation in the in Vitro Stimulation of Protein Synthesis in Human Fibroblasts by Ultrasound. Ultrasound Med Biol 4(4):**343-351**

Welgus HG, Jeffrey JJ, Eisen AZ (1981) Human Skin Fibroblast Collagenase. Assessment of Activation Energy and Deuterium Isotope Effect with Collagenous Substrates. J Biol Chem 256(18):**9516-9521**

White SR, Williams P, Wojcik KR, Sun S, Hiemstra PS, Rabe KF, Dorscheid DR (2001) Initiation of Apoptosis by Actin Cytoskeletal Derangement in Human Airway Epithelial Cells. Am J Respir Cell Mol Biol 24(3):**282-294**

Wild JJ (1950) The Use of Ultrasonic Pulses for the Measurement of Biologic Tissues and the Detection of Tissue Density Changes. Surgery 27(2):**183-188**

Wilder-Smith OH, Arendt-Nielsen L, Gaumann D, Tassonyi E, Rifat KR (1998) Sensory Changes and Pain after Abdominal Hysterectomy: A Comparison of Anesthetic Supplementation with Fentanyl Versus Magnesium or Ketamine. Anesth Analg 86(1):**95-101**

Williams AR (1983a) Ultrasound : Biological Effects and Potential Hazards. Academic, London. **pviii,321p.**

Williams AR (1983b) Ultrasound Biological Effects and Potential Hazards. New York Academy Press. **p126**

Wiltink A, Nijweide PJ, Oosterbaan WA, Hekkenberg RT, Helders PJ (1995) Effect of Therapeutic Ultrasound on Endochondral Ossification. Ultrasound Med Biol 21(1):**121-127**

Wood RW, Loomis AL (1927) The Physical and Biological Effects of High Frequency Sound Waves of Great Intensity. Philosophical Magazine 4:**417-436**

Woon Tiong A, Changhong Y, Jie C, El-Bialy T, Doschak M, Uludag H, Ying T (2008) System-on-Chip Ultrasonic Transducer for Dental Tissue Formation and Stem Cell Growth and Differentiation Circuits and Systems, 2008. ISCAS 2008. IEEE International Symposium on, pp 1818-1821

Wu J, Nyborg WL (2008) Ultrasound, Cavitation Bubbles and Their Interaction with Cells. Adv Drug Deliv Rev 60(10):**1103-1116**

Wu KY, Hong SJ, Huang HT, Lin CP, Chen CW (1999) Toxic Effects of Mitomycin-C on Cultured Corneal Keratocytes and Endothelial Cells. J Ocul Pharmacol Ther 15(5):**401-411**

Yamashiro T, Zheng L, Shitaku Y, Saito M, Tsubakimoto T, Takada K, Takano-Yamamoto T, Thesleff I (2007) Wnt10a Regulates Dentin Sialophosphoprotein Mrna Expression and Possibly Links Odontoblast Differentiation and Tooth Morphogenesis. Differentiation 75(5):**452-462**

Yan SG, Huang LY, Cai XZ (2011) Low-Intensity Pulsed Ultrasound: A Potential Non-Invasive Therapy for Femoral Head Osteonecrosis. Med Hypotheses 76(1):**4-7**

Yang KH, Parvizi J, Wang SJ, Lewallen DG, Kinnick RR, Greenleaf JF, Bolander ME (1996) Exposure to Low-Intensity Ultrasound Increases Aggrecan Gene Expression in a Rat Femur Fracture Model. J Orthop Res 14(5):**802-809**

Yang RS, Lin WL, Chen YZ, Tang CH, Huang TH, Lu BY, Fu WM (2005) Regulation by Ultrasound Treatment on the Integrin Expression and Differentiation of Osteoblasts. Bone 36(2):**276-283**

Yang X, van der Kraan PM, Bian Z, Fan M, Walboomers XF, Jansen JA (2009) Mineralized Tissue Formation by Bmp2-Transfected Pulp Stem Cells. J Dent Res 88(11):**1020-1025**

Yano F, Kugimiya F, Ohba S, Ikeda T, Chikuda H, Ogasawara T, Ogata N, Takato T, Nakamura K, Kawaguchi H, Chung UI (2005) The Canonical Wnt Signaling Pathway Promotes Chondrocyte Differentiation in a Sox9-Dependent Manner. Biochem Biophys Res Commun 333(4):**1300-1308**

Young SR, Dyson M (1990) The Effect of Therapeutic Ultrasound on Angiogenesis. Ultrasound Med Biol 16(3):**261-269**

Yue PY, Leung EP, Mak NK, Wong RN (2010) A Simplified Method for Quantifying Cell Migration/Wound Healing in 96-Well Plates. J Biomol Screen 15(4):**427-433**

Zhang R, Cooper PR, Smith G, Nor JE, Smith AJ (2011) Angiogenic Activity of Dentin Matrix Components. J Endod 37(1):**26-30**

Zhang ZJ, Huckle J, Francomano CA, Spencer RG (2003) The Effects of Pulsed Low-Intensity Ultrasound on Chondrocyte Viability, Proliferation, Gene Expression and Matrix Production. Ultrasound Med Biol 29(11):**1645-1651**

Zhou S, Schmelz A, Seufferlein T, Li Y, Zhao J, Bachem MG (2004) Molecular Mechanisms of Low Intensity Pulsed Ultrasound in Human Skin Fibroblasts. J Biol Chem 279(52):**54463-54469**

APPENDIX

PUBLISHED WORK

- **Man J**, Shelton RM, Cooper PR, Scheven BA (2011) Low intensity ultrasound promotes proliferation and differentiation of odontoblast-like cells. J Endod. Submitted
- Scheven BA, **Man J**, Millard JL, Cooper PR, Lea SC, Walmsley AD, Smith AJ (2008) VEGF and odontoblast-like cells: stimulation by low frequency ultrasound. Arch Oral Biol. Feb;54(2):185-91

ABSTRACTS/CONFERENCES

- **Man, J**, Shelton RM, Cooper PR and Scheven BAA. In-vitro wound healing by odontoblast-like cells in response to ultrasound. International Association for Dental Research, Barcelona 2010
- **Man, J**, Shelton RM, Cooper PR and Scheven BAA. Scratch Wound Healing in Response to Ultrasound in Bone and Tooth Cultures. Festival of Research and Enterprise, University of Birmingham 2010
- **Man, J**, Shelton RM, Cooper PR and Scheven BAA. Therapeutic Ultrasound: A Potential Tool for Tooth Repair and Regeneration. Third Annual Research Poster Conference, University of Birmingham 2009
- **Man, J**, Shelton RM, Cooper PR and Scheven BAA. Therapeutic Ultrasound: A Potential Tool for Tooth Repair and Regeneration. Festival of Research and Enterprise, University of Birmingham 2008
- **Man, J**, Jones CE, Caldwell GM, Cooper PR, Shelton RM and Scheven BAA. Ultrasound Induction of Wnt signalling pathway in odontoblast-like cells. International Association for Dental Research – Pan European Federation, London 2008

- **Man, J**, Shelton RM, Cooper PR and Scheven BAA. Therapeutic Ultrasound: A Potential Tool for Tooth Repair and Regeneration. Second Annual Research Poster Conference, University of Birmingham 2007