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# *In vitro* models to study compressive strain-induced muscle cell damage

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**Abstract.** Skeletal muscle tissue is highly susceptible to sustained compressive straining, eventually leading to tissue breakdown in the form of pressure sores. This breakdown begins at the cellular level and is believed to be triggered by sustained cell deformation. To study the relationship between compressive strain-induced muscle cell deformation and damage, and to investigate the role of cell–cell interactions, cell–matrix interactions and tissue geometry in this process, *in vitro* models of single cells, monolayers and 3D tissue analogs under compression are being developed. Compression is induced using specially designed loading devices, while cell deformation is visualised with confocal microscopy. Cell damage is assessed from viability tests, vital microscopy and histological or biochemical analyses. Preliminary results from a 3D cell seeded agarose model indicate that cell deformation is indeed an important trigger for cell damage; sustained compression of the model at 20% strain results in a significant increase in cell damage with time of compression, whereas damage in unstrained controls remains constant over time.

## 1. Introduction

Animal studies of soft tissues under mechanical load have shown that skeletal muscle tissue is highly susceptible to sustained compression, eventually leading to tissue breakdown in the form of pressure sores [6,11]. This breakdown begins at the cellular level with disintegration of contractile proteins and damage to the cell membrane and nucleus, followed by inflammatory reactions [2,8]. Although it is clear that both the duration and magnitude of compression affect cell damage, the mechanobiological pathways whereby tissue compression leads to cell damage remain to be elucidated. To date, theories have mainly focussed on cell metabolism [6,8,12], whereas the direct effects of cell deformation due to compressive straining have been neglected. Cell deformation, however, triggers a variety of effects such as altered membrane stresses, volume changes and cytoskeletal reorganisation, which may be involved in early cell damage. It has been shown that the response of muscle cells to deformation during tensile or shear straining is crucial to cellular degeneration or adaptation [5,13] and a comparable response might be expected for compressive straining. We therefore hypothesise that compressive strain-induced cell deformation is an important factor in the breakdown of muscle tissue during pressure sore development.

As it is impossible to examine the effects of cell deformation independently of other factors in *in vivo* muscle tissue, *in vitro* systems involving cultured cells need to be employed. Like many cell types, skeletal muscle cells cannot be conceived as isolated structures. They are well organised in parallel bundles, surrounded by extracellular matrix, to form a functional tissue. Hence, it is likely that muscle cell

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deformation is influenced by cell–cell interactions, cell–matrix interactions, and three-dimensional tissue configuration. To study the relationship between muscle cell deformation and cell damage in response to compressive straining a hierarchical approach is adopted, involving the application of complementary *in vitro* models, ranging from single cells to monolayers and three-dimensional tissue analogs under compressive straining. The present paper focuses on the description of these models and gives some preliminary results.

## 2. Model systems

### *2.1. Muscle cell culture*

All models involve the established C2C12 mouse skeletal myoblasts line (ECACC, UK). Culture media and additives are obtained from Biochrom AG (Germany), unless stated otherwise. The myoblasts are seeded at low densities and grown in monolayer culture. Every 3 days they are fed with fresh growth medium consisting of high-glucose DMEM, supplemented with 200 mM stable L-glutamine, 15% (v/v) Fetal Bovine Serum, 1% (v/v) non-essential amino acid solution, 20 mM HEPES and a gentamicin antibiotic solution at 5000 IU/5 mg per ml. The myoblasts are maintained in continuous passage using trypsinisation of subconfluent cultures ($\sim$70–80% confluency, $\sim 6 \times 10^4$ cells cm$^{-2}$). To induce fusion and myogenic differentiation of the cells into multinucleated, rod-shaped myotubes, confluent cultures are fed with differentiation permissive medium, consisting of high-glucose DMEM plus 2% (v/v) Horse Serum [1]. Alternatively, differentiating cultures are kept in serum-free medium (ICN Biomedicals, UK) for future biochemical analyses of cell damage [7]. The differentiation medium is replaced every 3 days.

### *2.2. Single cell and monolayer models*

Single myotubes (diameter: 10–30 $\mu$m, length: 150–450 $\mu$m) are transversely loaded in unconfined compression using a specially designed cell compression device (Fig. 1). In brief, the device consists of a stainless steel frame that rests on the stage of an inverted microscope, and a small wedge-shaped glass tip for compression of the cell in vertical direction. The tip is attached to a force transducer (model 406A, Aurora Scientific Inc., Ontario) and can be positioned in $x$, $y$, and $z$ directions using three micromanipulators (M-111.1.DG, Physik Instrumente, Germany) and a nano-positioning system (Nanocube P-611.3S, Physik Instrumente). The accuracy of the force transducer is 10 nN, whereas the position of the tip can

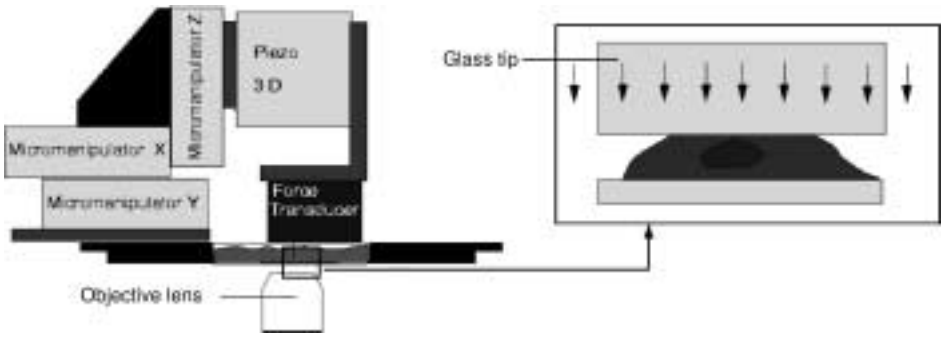


Fig. 1. Schematic representation of single cell compression device.

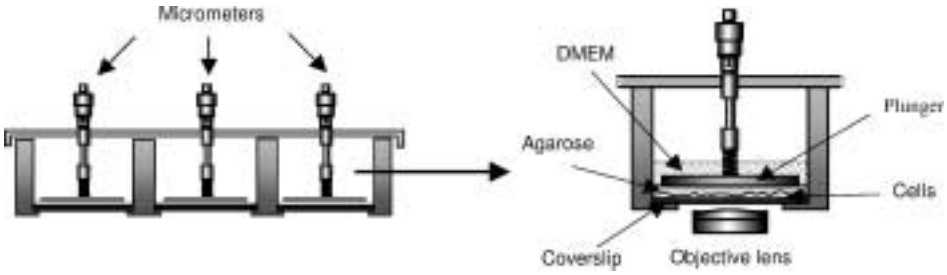


Fig. 2. Schematic representation of test rig for compression of muscle cell monolayers.

be adjusted at intervals of 5 nm for each direction. The whole setup is controlled by the software package Labview (National Instruments, Texas) that is also used for data recording. To prevent dehydration and shrinkage of the myotubes, measurements are performed in a bath of differentiation medium. For long-term experiments the device is equipped for control and regulation of temperature and $CO_2$. Three-dimensional cell deformation as well as structural damage to the myotubes is monitored by light and confocal laser scanning microscopy (CLSM) using an Axiovert 100 microscope equipped with a LSM 150 confocal system (Carl Zeiss BV, The Netherlands). For the latter application the cells are routinely stained with the viable probes Calcein-AM or Cell Tracker Orange (Molecular Probes BV, The Netherlands). To investigate the influence of adherent cells on compressive strain-induced cell deformation and damage the cell compression device is supplied with a larger tip and experiments are performed on monolayers of parallel-aligned myotubes.

For biochemical assays and statistical analyses of cell damage due to compressive straining relatively large numbers of cells, typically in the order of $1 \times 10^5$, are required. For this purpose a system for compression of multiple large monolayers is developed (Fig. 2). Myotube monolayers are grown on coverslips (diameter: 23 mm) at the bottom of a self-fabricated 6-well cell culture plate and compressed with porous glass plungers that can be graduated by micrometer heads at intervals $<0.01$ mm. To avoid influencing cell damage due to contact with the plunger, the monolayers are covered with a 1 mm thick layer of 3% low gelling temperature agarose (Type VII, Sigma-Aldrich Chemicals, The Netherlands). The culture plate can be positioned on the stage of a CLSM for the induction and periodical assessment of cell deformation. Otherwise, the culture plate is placed within an incubator. Cell damage is quantified periodically from viability assessments and evidence of membrane and/or nuclear damage using histology and biochemical analyses [3,10].

### *2.3. Three-dimensional models*

Three-dimensional tissue analogs involve cell seeded agarose constructs and tissue engineered muscle specimen. The cell seeded agarose system was developed in collaboration with the IRC in Biomedical Materials, Queen Mary, University of London and has recently been described [3]. To review, myoblasts are seeded in 3% low gelling temperature agarose at a density of $2 \times 10^6$ cells/ml. After gelling the suspension is cored into 5 mm diameter $\times$ 5 mm height cylindrical constructs, which are incubated in growth medium for 4 days, followed by an 8-day culture period in differentiation medium. By then the constructs contain a mixture of myoblasts and myotubes at a final cell concentration of approximately $1 \times 10^6$ cells/ml. A specially designed cell straining device consisting of an incubator within the loading frame of a testing machine [9] is used to subject the constructs to various unconfined compression

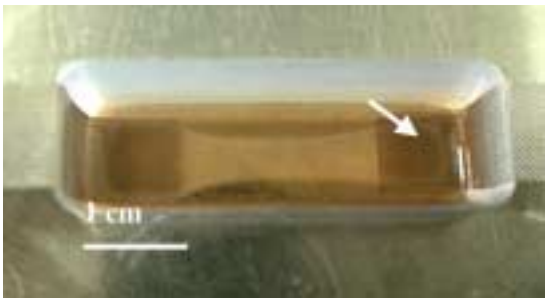


Fig. 3. Tissue engineered skeletal muscle in polycarbonate mould. The arrow indicates one of the grids, to which the tissue is attached.

regimens, whereas a test rig on the stage of a CLSM is used to assess cell deformation upon construct compression [3].

Since agarose does not provide anchor points for cell attachment, most muscle cells adopt a spherical morphology. Therefore, tissue engineered specimen consisting of parallel-aligned myotubes surrounded by an extracellular collagen/Matrigel matrix are produced to mimic the architecture of muscle tissue (Fig. 3). The production of the engineered muscle specimen is based on the work of Vandenburgh et al. [12], although protocols are slightly modified. In brief, myoblasts are seeded within a suspension of collagen solution (1.6 mg/ml) and Matrigel (6 : 1 v/v%, ICN Biomedicals) at a density of $4 \times 10^6$ cells/ml. The suspension is then plated in sterilised rectangular polycarbonate moulds, equipped with two stainless steel grids for anchorage of cells and gel, and incubated in growth medium. After incubation the gel starts contracting, thereby producing internal stresses to which the cells respond by aligning parallel to the long axis of the mould. Further alignment and myogenic differentiation is induced by switching to differentiation medium after 3 days in culture. The engineered specimen are transversely loaded in unconfined compression using a test rig, graduated by a micrometer head, and kept within an incubator. Similar to the previous systems this rig can be positioned on the stage of a CLSM to assess cell deformation.

For both the cell seeded agarose model and the tissue engineered model cell damage in strained and unstrained constructs is quantified from viability assessments and histological or biochemical analyses.

## 3. Results

All model systems have been characterised regarding reproducibility and cell viability. The single cell model is obviously easy to reproduce, whereas the tissue engineered muscle analogs are difficult to reproduce and show significant variations in viability with time of culture. Nonetheless, all model systems can be used to study the effects of sustained compression regimens (0–24 h) since they remain viable for at least 6 days and cell damage due to compressive straining is normalised to values in well-characterised unstrained controls obtained under identical culture conditions.

Initial compression experiments on cell seeded agarose constructs show that cell deformation follows gross construct strain up to 20% compression, while further straining causes buckling of the cell membrane in the majority of the cells. Moreover, sustained compression at 20% strain results in a significant increase in cell damage with time of compression. Histological observations demonstrated percentage values of cell damage ranging from 43% after 1 h of compression to 96% after 24 h of compression, whereas cell damage in unstrained controls remained constant over time (29%). Viability measurements

confirmed the results. These findings indicate the importance of sustained cell deformation as a promoting factor in cell damage.

## 4. Conclusion

To investigate the relationship between compressive strain-induced muscle cell deformation and damage a range of *in vitro* models is being developed. Such models offer a potential for well-characterised specimen and enable improved control of experimental conditions with less stringent ethical considerations than *in vivo* models. Although the described models are limited representations of *in vivo* muscle, they mimic different levels of tissue complexity, which are very useful to test our hypothesis and to study the influence of adjacent cells, the extracellular matrix and 3D tissue architecture on cell deformation and damage. Our initial results demonstrate that compressive strain-induced cell deformation is indeed an important trigger for cell damage. However, the relative effects of magnitude and duration of deformation remain to be elucidated. Future studies should thus involve different clinically relevant straining regimens, defined in terms of magnitude (0–40% strain) and duration (0–24 h), to find general threshold levels for cell damage. In order to extrapolate these results to the clinical situation related studies from our group focus on *in vivo* experiments using animal models [2]. Moreover, numerical models are being developed to predict the amount and location of cell damage in response to tissue compression. The latter involve the application of multiscale finite element modelling to couple gross macroscopic tissue strains to the complex micro-mechanical conditions at the cellular level [4].

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