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Compression Induced Cell Damage in Engineered Muscle Tissue: An *In Vitro* Model to Study Pressure Ulcer Aetiology

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Abstract—The aetiology of pressure ulcers is poorly understood. The complexity of the problem, involving mechanical, biochemical, and physiological factors demands the need for simpler model systems that can be used to investigate the relative contribution of these factors, while controlling others. Therefore, an *in vitro* model system of engineered skeletal muscle tissue constructs was developed. With this model system, the relationship between compressive tissue straining and cell damage initiation was investigated under well-defined environmental conditions. Compression of the engineered muscle tissue constructs revealed that cell death occurs within 1–2 h at clinically relevant straining percentages and that higher strains led to earlier damage initiation. In addition, the uniform distribution of dead cells throughout the constructs suggested that sustained deformation of the cells was the principle cause of cell death. Therefore, it is hypothetised that sustained cell deformation is an additional mechanism that plays a role in the development of pressure ulcers. © *2003 Biomedical Engineering Society.* [DOI: 10.1114/1.1624602]

Keywords—Decubitus, Pressure ulcers, Cell viability, Cell damage, Cell deformation, Confocal microscopy, Fluorescent dyes, Tissue engineering.

INTRODUCTION

Soft tissues are highly sensitive to prolonged compressive loading, eventually leading to tissue breakdown in the form of pressure ulcers.[9,20] This loading is induced by the body weight that compresses skin and underlying fat and skeletal muscle tissue between a supporting surface and bony prominences. Pressure ulcers can also occur from orthoses and prostheses which apply corrective forces that compress the soft tissue composite. Pressure ulcers are painful and difficult to treat. Moreover, they put a heavy burden on health care budgets.

Pressure ulcers can develop either superficially or from within the deep tissue depending on the nature of loading.[9,10,15,17,25,33] Several studies have shown that skeletal muscle tissue is particularly susceptible for pressure ulcer development.[9,15,22,25] Since ulcers that initiate in muscle tissue are generally more extensive and develop at a faster rate than superficial ulcers,[2] this study focuses on damage initiation in skeletal muscle tissue.

It is clear that loads acting on the skin are transferred to the underlying muscle tissue, changing the local cellular environment. At this level, cell damage starts with signs of disintegration of contractile proteins and damage to the membrane and nucleus, followed by inflammatory reactions.[4,17,20] However, it is not fully understood which changes in the local biomechanical and biochemical environment can initiate cell damage. Consequently, the relationship between local cell damage and the load magnitude and time is unclear.

Several theories have been proposed, to explain how compressive tissue straining leads to local cell damage. These theories focus on different functional units of the tissue namely the vessels, interstitium, and the cells. The most commonly adhered theory is that compressive straining causes occlusion of capillary blood flow, resulting in local ischaemia.[9,10,17] Apart from local ischaemia, other mechanisms are likely to be involved which either directly induce cellular damage or increase the vulnerability of the cells. Reddy[23] hypothesized that occlusion of the lymphatic system leads to accumulation of metabolic waste products[22,23] which leads to cell damage. Another theory relates to reperfusion damage after loading of the tissue.[19] Finally, it was proposed that sustained cell deformation forms an additional mechanism for cell damage eventually leading to pressure ulcers.[4,6]

Attempts to unravel the precise pathways of pressure ulcer development have been impeded by a lack of reproducible model studies of the clinical condition. So far, animal models from different species have been used.[9–11,15,16,20,25] In these studies, an external load was applied to the skin with an indenter that compresses skin and underlying tissues. These studies focused on establishing threshold values for tissue damage in terms of externally applied pressure and time of compression. More recently, Bosboom[3] and co-workers examined both

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the amount and location of damage in a rat model using magnetic resonance imaging (MRI) techniques.

The animal studies have provided most of the present knowledge on the aetiology of pressure ulcers, however, they are less suitable for unraveling the mechanisms that lead to local cell damage. This is because these studies do not allow independent control or observation of the proposed mechanisms for cell damage since all functional units of the tissue are affected by external load application.

In vitro skeletal muscle models may provide a complementary method for studying mechanisms for cell damage.[5,6] In addition, they are useful to establish relationships between cell damage as a function of time and magnitude of loading. The first objective of this study was to develop such an *in vitro* model of skeletal muscle tissue. A second objective was to study damage evolution in time for different clinically relevant straining regimes. For this purpose, a novel compression device was developed. Finally, by quantification and localization of viable and dead cells using previously developed image analysis techniques[7] it is possible to test the hypothesis that cell deformation forms an additional mechanism for cell death, which may play a role in the onset of pressure ulcers.

MATERIALS AND METHODS

Engineered Skeletal Muscle Tissue Constructs

Constructs of skeletal muscle myotubes embedded in a gel matrix were developed by modification of protocols from Vandenburgh and co-workers.[30] C2C12 mouse myoblasts [European Collection of Cell Cultures (ECACC), Salisbury, UK] were cultured in growth medium consisting of DMEM high glucose, 20% fetal calf serum, 2% hepes, 1% nonessential amino acids, and 1% gentamycin (all purchased form Biochrom AG, Berlin, Germany). The cells were replated at 70% confluency to avoid premature differentiation. At passage 20 the cells were trypsinized and resuspended in growth medium at a concentration of 1.25×10^6 cells/ml. This cell suspension was gently but thoroughly mixed with an ice-cold mixture of rat tail collagen type I solution, containing 3.2 mg collagen (Sigma, St. Louis, MO) per ml 0.2 (v/v%) acetic acid, neutralized with 0.5 M NaOH according to methods described by Bell.[1] Furthermore, growth factor reduced Matrigel (BD Biosciences Discovery Labware, Lincoln Park, NJ) was added to this mixture. The ratio of collagen solution cells in growth medium matrigel was 6:4:1 (v/v%) which implies that each construct initially contains approximately 3.4×10^5 cells.

The gel/cell mixture was carefully added to six-well culture dishes (750 μl per well), forming disk-shaped constructs. The constructs were kept at room temperature for 10 min to increase the viscosity of the mixture.

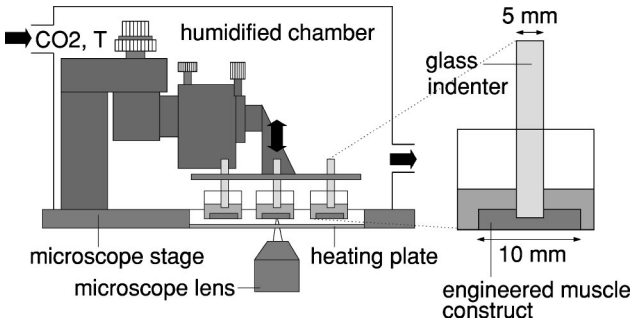

FIGURE 1. Schematic representation of the construct compression device (side view), with a magnification of the construct which is compressed by a glass indenter. The whole setup is covered by a custom made incubator, with an inlet for humidified, heated and CO_2 controlled air.

Thereafter, the constructs were carefully transported to a humidified incubator. In the incubator, the constructs were allowed to attach to the culture wells for 6 h, after which they were overlaid with fresh growth medium. Every 24 h, the constructs were rinsed and fed with 3 ml fresh growth medium. From day 3 onwards, growth medium was replaced by fusion medium, which was added to the wells to stimulate fusion and differentiation of the myoblasts into multinucleated, elongated myotubes. The fusion medium consisted of DMEM high glucose, 2% horse serum, 1% gentamycin, 1% nonessential amino acids and 2% hepes.

Compression Device

A compression device was developed to simultaneously compress six muscle constructs with circular, impermeable glass indenters, each of diameter 5 mm. The compression device is based on a micropipette manipulator system (Fig. 1). A stainless steel plate which holds the indenters was connected to the fine vertical positioning carriage of the manipulator. Two coarse spindles were used to position the glass indenters above the layers. A fine micrometer spindle, with an accuracy of 10 μm, was used for gently lowering the plate and thus applying the strain onto the constructs. The whole setup was mounted on a computerized $x-y$ stage with a control unit (MCU 28, Zeiss, Germany) that was mounted on a confocal laser scanning microscope (Zeiss LSM 510). The moment of contact between the indenter and the constructs could be easily determined by visualizing the indenter and the top of the constructs using transmission microscopy. During compression of the constructs, air temperature, CO_2 and humidity was controlled by an incubator system (Tempcontrol 37-2, CTI-controller 3700 purchased from PeCon GmbH, Germany). This incubator system circulated the air in a custom made plastic chamber that covered the setup. The culture medium temperature was stabilized at 37° using

a custom made six-well heating plate based on a printed circuit board with a thermocouple sensor (TCMK-S12, TMC Instruments, The Netherlands) and a control unit (West N2300/Y1200, TMC Instruments, The Netherlands).

Compression Protocol

For each experiment, 10 day old constructs were used whereby day 1 is defined as the moment where the cell/gel mixture was added to the wells. The initial height of these constructs was determined from axial z-stack scans of cells stained with CellTracker™ Green (see next section). For each construct, the mean height was determined by averaging measured heights at nine randomly chosen locations below the indenters. Based on these height measurements, the glass indenters were lowered by prescribed amounts equivalent to gross compressive strains of 30% and 50%, respectively. These straining levels were based on MRI studies of compressed tissues near bony prominences.[28] As a control, also unloaded constructs and constructs with only the glass indenters touching the construct were monitored. These latter controls were used to study the effect of the impermeable glass indenters, since the impermeable glass indenters on top of the layer may prevent culture medium to reach the constructs from this side. This may result in a reduced nutrition supply to the cells below the indented area. For the latter constructs, the indenters were carefully lowered while the moment of contact was determined by transmitted light microscopy. This resulted in a negligible initial strain. For each strain regime $n=6$ constructs were used except for the 30% strain experiment, for which $n=3$ was used.

Characterization of the Constructs

Morphology of the cells within each construct was regularly examined using a conventional transmission light microscope (Zeiss). For monitoring the viability of the cells and measuring layer heights, the fluorescent probes CellTracker™ Green (CTG) and propidium iodide (PI) were used (both purchased from Molecular Probes, USA). CTG stains the cytoplasm of living cells and was used for visualisation of the cells whereas PI was used to identify dead cells. In all experiments an optimum probe concentration of 10 μM (CTG) and 5 μM (PI) was used.[7] The constructs were loaded with CTG for 5 min, rinsed twice with poly butene sulfone, and incubated for 30 min in a humidified incubator. After rinsing again, PI dissolved in fresh growth medium was added to each well (3 ml per well) after which the six-well plate was mounted onto the microscope stage. A $10\times$ plan apochromatic lens (numerical aperature$=0.3$) was used to obtain fluorescence images at a 512×512 pixel resolution. For all measurements an optical slice thickness of 10 μm was chosen.

To determine the viability across the area below the indenter, 25 tile-scan fluorescence images (5×5 tiles) were taken, covering an area of 5 mm square below each indenter. Positioning of the microscope stage was performed fully automatically by programming the computerized $x-y$ stage (Zeiss Multi Time Series software). For each strain regime, images were taken from the central horizontal section of the constructs at $t=0$, 1, 2, 4, 6, and 8 h. The four corner tiles which were situated only partly below the circular indenter were excluded from data analyses. Dead cell numbers were quantified by automated counting of nuclei from PI images using previously developed image analysis software[7] written in Matlab (The Matworks, Natick, MA). Since the constructs contain multinucleated myotubes, this is not exactly equal to the number of dead cells, since myotubes may be only partly damaged. Therefore, in the remainder of this paper, ''dead cells'' should be interpreted as the number of PI stained nuclei. Percentage cell damage was defined by the number of PI stained nuclei normalized by the average total number of nuclei within the central horizontal confocal plane of the compressed construct. To determine this total cell number, at the end of the experiments 2 ml of 70% alcohol was added to each well which rapidly killed all the cells. 5 min after alcohol addition, tile scans were taken to determine the total number of PI stained nuclei.

Data Analyses

The percentages dead cells were analyzed as a function of time for different straining regimes using SPSS (SPSS, Chicago, IL). Nonparametric Kruskal–Wallis tests were performed to investigate whether dead cell numbers significantly increased with time for each straining regime. Post hoc comparisons (Mann-Whitney U) were performed between different moments in time at a given straining percentage. Moreover, differences in mean dead cell percentages were analyzed for different straining regimes at a given time. To investigate whether cell damage varied across the area below the indenter, dead cell percentages in two selected areas of the tile-scan images were compared using pairwise Mann–Whitney U tests. Unless stated otherwise, the $p<0.05$ level of significance was used.

RESULTS

Engineered Skeletal Muscle Tissue Constructs

Figure 2(a) shows an example of an engineered muscle tissue construct. After addition of the gel-cell mixture to the wells, the constructs attached to the cul-

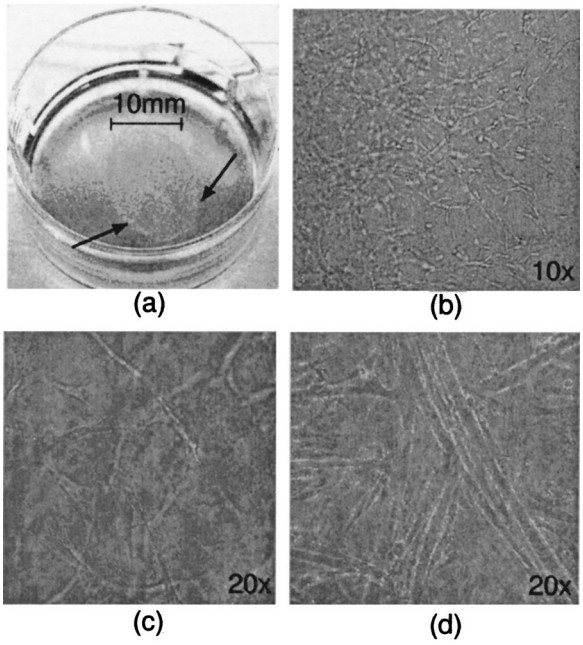

FIGURE 2. (a) Disk-shaped engineered muscle tissue construct with slightly curled edges (arrows). (b), (c), (d) Transmission microscopy images showing: (b) myoblasts at the edge of the engineered muscle tissue construct (day 1), (c) a network of connected myoblasts within the construct (day 3), and (d) a branched network of elongated myotubes (day 8).

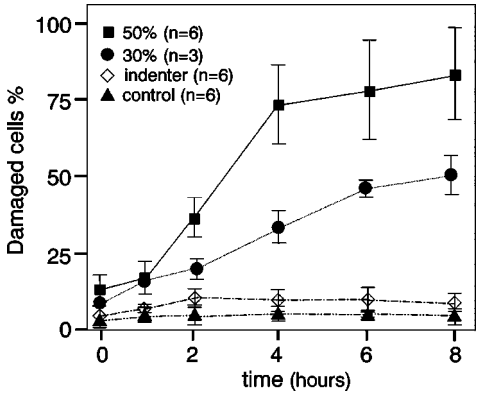

FIGURE 3. Percentage dead cells as a function of time for different straining regimes. Data is presented as mean ± scanning electron microscopy.

ture wells within 3–6 h. The attachment was mediated by cells that are both in contact with the well substratum and the gel. During the first 2 h, fluid was squeezed out of the constructs and the diameter decreased from approximately 15 to 10 mm due to gel contraction. After attachment to the substratum, the diameter of the constructs remained constant over time. The edges of the constructs were slightly curled inwards [see arrows in Fig. 2(a)]. Some cells at the edges of the construct that were not fully entrapped by the gel proliferated and formed a monolayer connected to the edges of the construct [Fig. 2(b)]. These cells additionally supported the construct's attachment to the well. Nevertheless, occasionally a construct detached from the well. When this happened, the construct was excluded from further experiments.

During the first 3 days, the myoblasts rapidly proliferated, forming a multilayered, three-dimensional network of connected cells [Fig. 2(c)]. After replacement of the growth medium by fusion medium on day 4, the myoblasts fused into a branched network of multinucleated, elongated myotubes [Fig. 2(d)]. The myotubes showed spontaneous contractions after 8 days. After 10 days in culture, the engineered skeletal muscle constructs exhibited approximately 70% differentiated cells with an overall viability of more than 95%. The constructs could be kept in culture for at least 30 days without compromising viability.

Compression Experiment

The results of the compression experiments are summarized in Fig. 3. The unstrained controls are identified as ''control,'' whereas the constructs whose surfaces were in contact with a glass indenter causing a negligible initial strain, are identified with ''indenter.'' Time $t = 0$ h is defined as the moment at which the indenters were lowered onto the constructs.

The results show that the increase in dead cell percentages with time was significant for the constructs subjected to 30% and 50% strain ($p < 0.001$). At a 50% gross compressive strain level, 13.6% of the cells died immediately after strain application ($t = 0$ h). Cell death significantly increased between 1 and 4 h compression and remained nearly constant thereafter. Post hoc comparisons revealed that the differences in number of dead cells for the 50% strained constructs as compared to the control and indenter groups were significant for each moment in time. Constructs strained to 30% invoked 8.2% initial cell damage, followed by a more gradual increase between 2 and 6 h (Fig. 3). The number of dead cells for the 30% strained construct were significantly larger than the control and indenter groups at $t = 4-8$ h. Post hoc comparisons between the 30% and 50% straining regime revealed that the differences in mean dead cell numbers were significant at the $p < 0.05$ level between 4 and 8 h.

The indenter constructs showed an initial dead cell percentage of approximately 4.2%, with a small, nonsignificant increase between 1 and 2 h. Dead cell percentages in the control constructs remained constant at approximately 5% over time. Post hoc tests indicated that the differences in dead cell percentages between the con-

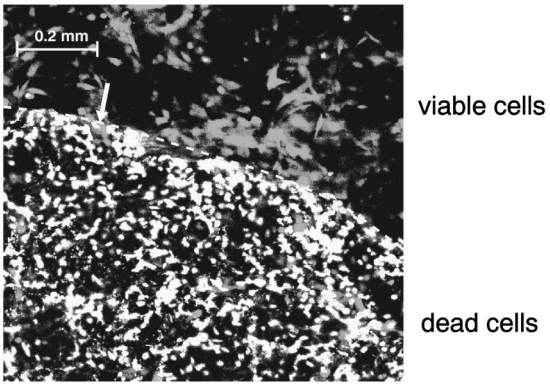

FIGURE 4. Confocal tile-scan image covering an area of 2 mm square at the periphery of the indenter. The PI stained nuclei indicated with the bright white dots are only present below the indenter as shown by a clear demarcation line between damaged and undamaged areas (see arrow).

trol and indenter group were not significant for each moment in time.

In the 30% and 50% strained constructs, dead cells were found to be highly localized below the indenter (Fig. 4). However, below this indenter, a random distribution of dead cells was found (Fig. 5). To investigate whether the percentage of dead cells depended on the location below the indenter, dead cell numbers at the center of the indenter were compared to dead cell numbers at the periphery of the constructs. This was achieved by analyzing two selected areas of tile-scan images from the constructs. The selected areas covered 1 mm square, located below the center of the indenter and a similar area at the periphery (see squares in Fig. 5, $t=4$ h). Pairwise comparisons revealed that at none of the time periods ($t=0,2,4,6,8$ h) there were significant differences in cell damage between the center and peripheral areas ($p>0.5$).

DISCUSSION

The mechanisms underlying the aetiology of pressure ulcers are not well understood. The complexity of the problem involving mechanical, biochemical, and physiological factors demands the need for simpler model systems that allow investigation of the relative contribution of individual factors while controlling others. For this purpose, a novel *in vitro* model of skeletal muscle tissue has been developed that could be compressed under well defined conditions.

The applicability of the model system was demonstrated by compressive straining of the constructs, leading to highly localized cell death at clinically relevant strains. Compressive straining induced cell death be-

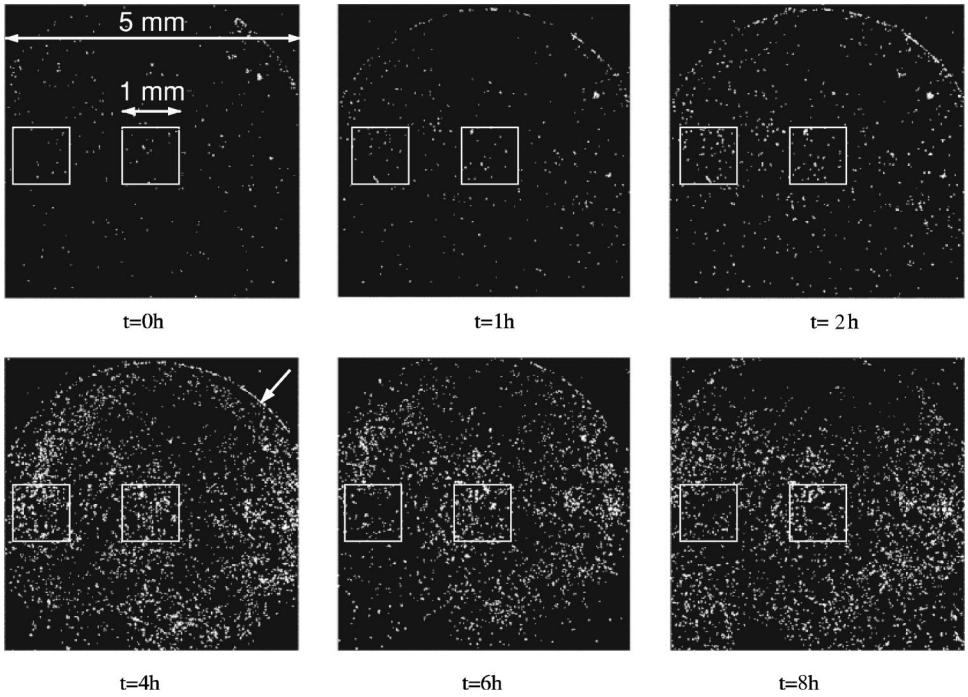

FIGURE 5. Tile-scan images, covering the indented area, which show the distribution of dead cells as a function of time for 50% compressed constructs. The white dots represent nuclei of dead cells. The edges of the circular indenter are clearly visible (see arrow at $t=4$ h) with only a limited number of nuclei visible away from the indenter area. The percentage dead cells gradually increases from $t=1$ to 4 h, but the distribution of dead cells remains uniform across the indented area for each point in time. The white squares indicate the selected areas that were analyzed for comparison of the percentages dead cells in the center and near the periphery of the indenter.

tween 1 and 2 h which is comparable to what has been reported in an *in vitro* model[6] and animal experiments on muscle tissue.[3,15,17] Dead cells were located below the indenters where a clear demarcation line was present that corresponds to the edge of the indenter and the inherent stress concentration effects. The results in Fig. 3 showed that at 30% and 50% straining, 8.2% and 13.6% of the cells immediately died, respectively. Presumably, this is a consequence of direct trauma to the cell membrane, caused by disruption or buckling of the membrane.[6] However, the vast proportion of the cells died between 1 and 4 h. A 30% strain level, led to smaller percentages dead cells and a delayed damage growth as compared to the 50% strain level.

The results agree with the well known pressure ulcer risk curve presented by Reswick[24] who found an inverse proportional relationship between load magnitude and time required for pressure ulcer development. With respect to clinical practice, the present results suggest that even a few hours of compression may cause severe damage to muscle tissue. Thus, although pressure ulcers may become visible at the skin after 1–2 weeks, the initiation may have occurred within a short period of compression. This endorses the viewpoint of several observers[26] who have suggested that pressure ulcers in fact often develop during the relative short period in operation rooms where, as a rule, less attention is paid to pressure reducing support systems.

The cause of cell damage could be a direct consequence of sustained cell deformation or from impaired transport of both metabolites and waste products within the construct. The present results, however, provide a number of arguments that suggest that sustained cell deformation invoked cell death. First, if impaired transport of both metabolites and waste products within the construct was the primary cause of cell damage, a gradient in damage might have been expected from the center towards the periphery of the construct. However, this was not seen in the results, presented in Fig. 5. In addition, the constructs with an indenter resting on them did not show an increase in damage over time. Apparently, blocking the diffusion pathways by the impermeable glass indenter on one side and the culture well bottom on the other side was not harmful to the constructs within this time frame. Even after 24 h, the impermeable glass indenters resting on the constructs did not increase cell death (data not shown). Since damage occurred within a few hours, transport of substrates to the cells within the constructs was apparently not a major trigger for cell death. Finally, it appeared that constructs subjected to both levels of compressive strain did not squeeze out all fluid below the indenter, since the PI dissolved in the growth medium could still reach the cells.

These findings suggest that the increase in cell damage with time was predominantly caused by sustained cell deformation. However, the mechanisms whereby cell deformation induces cell damage still remain to be elucidated. Presumably, cell deformation impairs diffusion of large molecules through the cell membrane. In addition, direct structural trauma to load bearing structures within the cell may play a role. Furthermore, it should be noticed that the compression of the constructs may have decreased the permeability across the whole indented area, thereby affecting the cell susceptibility for damage. Therefore, elucidating diffusion pathways of both small molecules and larger vital molecules will be subject of future work.

In the present study, the actual local cell deformations which result from the applied gross compressive construct strain were not quantified. In future, such measurements may be carried out by using a cytoplasmic stain, which can be visualised with the confocal microscope.[6,12,13] However, the random alignment of the cells hamper such measurements. In addition, the limited axial resolution of the confocal microscope at larger working distances and aberrations in the vertical direction inherent to confocal microscopy complicate quantification of cell deformations in the deeper tissue.[12,32]

Clearly, the local cell deformation state is a complex interplay between the externally applied gross compression and the prestress generated by the attachment to the well in combination with internal cellular forces acting on the gel. In addition, the local structural heterogeneity and the viscoelastic behavior of both cells and gel play an important role. Thereby, it should be noticed that the extracellular environment in the *in vitro* model differs from the *in vivo* situation. Consequently, the applied gross compressive strain may have resulted in cell deformations that exceed physiological conditions at which pressure ulcers occur, which may have led to an overestimation of cell damage in the *in vitro* experiments. Indeed, numerical simulations have shown that the local cell environment has a profound impact on the actual deformation sensed by the cells as a consequence of tissue compression.[8] In this particular study, it was shown that the typical microstructural organization of adult muscle tissue led to smaller cell deformations than a random cell distribution as is typical for the *in vitro* constructs used in the present study.

A limitation of the present experimental setup concerns the accuracy of strain application. In theory, the accuracy of the strain application is within 5 μm, however, in practice this accuracy will not be reached. This is due to the obliquity within a single culture well and the obliquity of the whole six-well plate that is mounted on the stage of the confocal microscope. The relative low bending stiffness of the commercially available six-well plates that were used in the present study further complicates accurate strain application. By careful alignment of the indenter surface with the bottom surface of the

well, it was attempted to minimize these inaccuracies.

Previously, our group developed an *in vitro* model consisting of muscle cells embedded in an agarose matrix.[6] One of the limitations of this model was that the cells in the agarose model adopted a spherical shape. The present model is more representative in the sense that the cells formed a branched network of contractile, elongated myotubes arranged in a three-dimensional multilayered network. In the past, various authors have attempted to engineer artificial muscle constructs either on a basement membrane[14] or in the form of three-dimensional constructs called organoids or myooids.[18,21,27] Provided that the cells within the gel are subject to internal stresses in the gel, the cells in such constructs contain specific muscular cytoskeletal proteins as shown by antibody labeling techniques.[29] Nevertheless, the myotubes in the present *in vitro* model will only proceed to primary myotube stage having a different phenotype from myofibers *in vivo*. In addition, the *in vitro* model lacks the typical three-dimensional organization with transverse mechanical connectivity. When interpreting the results this should be kept in mind since adult skeletal myofibres may behave differently. Future studies will focus on improving the representativeness of the engineered constructs by using cocultures of fibroblasts and myoblasts.

A difficulty in maintaining a lattice of cell populated gels is their propensity to detach from the substratum due to cell generated internal forces.[31] This problem can be solved by providing some form of artificial tendon that serves as attachment site.[29] Then, after some days in culture, the construct can detach from the well while being maintained between the artificial tendons. For the present study, such a system was less feasible since controlled compression and monitoring of such ''floating'' organoids is difficult. Therefore, the detachment problem was solved by suspending a low concentration of cells in the collagen-matrigel mixture which reassured that the contractile forces in the first few hours did not exceed the forces keeping the gel connected to the well. Thereby, the chosen cell concentration appeared to be critical. In a later stage, also cells that grew in a monolayer surrounding the disk-shaped construct supported the attachment to the well. The equilibrium of internal and external forces causes internal stresses in the gel which are required for long term maintenance of myotubes in the gel.[14,31] In this way, it was achieved to create constructs with an overall viability of more than 95%, which could be kept in culture for at least 1 month without compromising viability (data not shown).

Summarising, an *in vitro* model system was developed which aims at elucidating the pathways of pressure ulcer related muscle damage. This model allows studying relationships between compressive straining and cell death under well defined conditions. Results indicated that a significant increase in cell death can occur at clinically relevant strains within 1–2 h and that a higher strain led to earlier damage initiation. In addition, the present results suggested that prolonged cell deformation is an additional mechanism leading to cell damage which could play a role in the onset of pressure ulcers.

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