Evaluation of an in-vitro model to study pressure induced skeletal muscle damage related to pressure sores

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Abstract

An experimental model system and a compression device have been developed which enable study of the hypothesis that cell deformations directly lead to cell damage related to pressure sores in muscle tissue. In this study the newly developed model system was characterized and the consequences of sustained cell deformation in terms of local cell damage were studied. Tissue engineered muscle layers were made. They consisted of myoblasts in a collagen I–Matrigel mixture, which were cultured in growth medium for 3 days. Subsequently, the cells were allowed to fuse into multinucleated myotubes for 8 days in differentiation medium. To characterize the layers the height has been measured and the cell distribution throughout the layers has been measured. The ten day old layers had an equal height of 450 µm and in all the layers the cells were distributed in the same way, the top layers contained the most cells. A compression device has been developed with which a gross compressive strain 20% has been applied to the tissue engineered muscle layers. The cell damage significantly increased compared to two control groups. Since predisposing factors for cell damage, such as oxygen and nutrient supply to the layers were equal for strained and unstrained layers, the deformation of the layers and associated cells was considered as the trigger for cell damage.
Introduction

Pressure sores are localized areas of degenerated skin and underlying soft tissues caused by sustained mechanical loads. They are painful for patients and generally cure very slowly. Pressure sores prevalence is high; in the USA, between 2.3% and 28% percent of the patients in acute care hospitals and nursing homes develop some sort of pressure sores. Clearly, this high prevalence puts a heavy burden on the health care budget caused by the expensive prevention measures and prolonged hospitalization periods.

Pressure sores prevention is difficult and therefore the high prevalence prevails. A few explanations can be given. First, the most severe pressure sores initiate at deeper tissue layers and become visible when prevention is too late. Second, objective criteria for developing pressure sores are difficult to provide due to a large number of patient-related and environmental factors that predispose and affect tissue susceptibility for this development. Such contributing factors include heat, moisture, incontinence, malnutrition and altered levels of consciousness. Perhaps the most crucial problem for adequate prevention is the lack of insight into the aetiology of pressure sores. Although generally accepted that external mechanical loads are the main trigger for pressure sores, the underlying mechanisms are poorly understood.

In literature three theories have been proposed to explain the onset of pressure sores. The most commonly adhered theory is that external loading of the muscle tissue causes occlusion of capillary blood flow, resulting in ischemia followed by tissue necrosis. However there is some evidence that lack of oxygen supply is probably not the sole determinant for tissue damage as was long believed. A second theory involves the impairment of lymphatic drainage and/or interstitial fluid flow due to external loading. More recently, it was proposed that cell deformations directly lead to cell degeneration (Bouten et al., 2001; Bosboom et al., 2001).

The majority of studies on pressure sores that attempt to establish load threshold values have focused on external pressure as a global threshold below which pressure sore will not occur. This is often carried out using animal models. However, due to the complexity of the underlying tissue, such global loads may not be indicative for local loads within the tissue and thus to the onset of pressure sores. Since damage initiates at the cellular level, it seems logical to investigate changes in the local conditions that are caused by the application of an external load. However, there is still much debate on which local factors (i.e. occlusion of blood flow, impairment of lymphatic flow or direct cell deformation) are responsible for cell damage, research should be focused on identifying determinants for cell damage under external loading conditions. The widely used animal models are less applicable to study the hypothesis, since it is not possible to distinguish the components that may play a role.

In this study an in-vitro experimental model system has been developed which consists of engineered skeletal muscle layers. This model was chosen for its ability to test different hypothesis on the onset of damage under controlled environmental conditions. Compared to the study of Bouten et al, 2001, in which cells in an agarose gel were strained, this model system consists of muscle cells in a collagen-matrigel mixture and therefore is a more accurate representation of the in-vivo muscle tissue. Furthermore, a compression device has been developed to simultaneously compress six tissue engineered skeletal muscles.

The first aim of the present study was to characterize the newly developed model system. This was done by measuring the height of the layers and the cell distribution throughout the layers. The second aim was to study the consequences of sustained cell deformation in terms of local cell damage. Therefore a compression experiment has been carried out in which the cells were stained and then a strain was applied to the tissue engineered skeletal muscle layers. The layers were monitored with a confocal laser scanning microscope and the viability was calculated using a viability assay. This way local information about cell damage can be obtained.
Chapter 1: Materials and methods

1.1 Tissue Engineered Skeletal Muscle
For both testing and application of the method, the C2C12 mouse myoblasts cell line was used (ECACC, UK). The cells were cultured in growth medium (GM) consisting of DMEM High Glucose with 20% Fetal Calf Serum, 2% Heps, 1% non-essential amino acids, and 1% gentamycin. Fusion medium (FM) consisting of DMEM High Glucose, 2% Horse Serum, 1% gentamycin, 1% non-essential amino acids and 2% Heps, was used to stimulate fusion and differentiation of the myoblasts into myotubes. All media constituents were purchased from Biochrome KG (Germany).

Layers of skeletal muscle myotubes embedded in a gel matrix were developed by modification of a protocol developed by Vandenburgh. C2C12 cells were cultured in 25 cm² and 75 cm² Nuclon culture flasks (Merck, Germany), thereby replating the cells at 70% confluency to avoid differentiation. C2C12 skeletal muscle myoblasts, passage 20, were trypsinized and resuspended in GM at a concentration of 1.25 x 10⁶ cells per ml GM. This cell suspension was gently mixed with an ice-cold mixture of rat tail collagen I (Sigma) and Matrigel (Becton Dickinson) such that the ratio cells to collagen mixture was 4 to 6 (v/v%). The collagen solution consists of 32 mg collagen per ml acetic acid, neutralized with 0.5 M NaOH. The ratio collagen to Matrigel was 6 to 1 (v/v%). The gel/cell mixture, 750 µl per well, was carefully added to a 6-well culture dish. The layers were allowed to gel for five to ten minutes at room temperature after which they were transported to a humidified incubator. The layers were allowed to further attach to the culture wells for two hours in the incubator, after which they were overlaid with GM. The relative low initial cell concentration assured that the forces, generated by the cells within the gel, never exceed the attachment forces to the well. The first two days, every 24 hours, the layers were rinsed and fresh GM was added to the wells. From day 3 forward, FM was used instead of GM to induce differentiation of the myoblasts. FM was refreshed every 24 hours.

1.2 Characterization of the layers
For characterization of the layers (layer height and cell distribution) and monitoring the viability of the cells, the cells were stained. The staining is based on the combination of the fluorescent probes CellTracker Green (CTG), staining vital cells, and Propidium Iodide (PI), staining the DNA of dead cells. Both probes were purchased from Molecular Probes (USA). In all experiments an optimum probe concentration of 10 µM was used (Breuls et al, submitted). Before staining, the muscle layers were first rinsed twice with PBS. Then the layers were loaded with CTG for 5 minutes and rinsed again, after which the PI was added. All samples were visualized with a Zeiss 510 confocal laser scanning microscope. CTG is excited with a 25 mW Argon laser at 488 nm. The emitted light has a peak near 516 nm and is collected using the bandpass filter BP 505-530. PI is excited with a 1 mW Helium-Neon laser at 543 nm and the emitted light is collected after passing the long pass filter LP 585. A multitrack scanning mode was employed to exclude cross-talk. A 10x 0.3 NA plan apochromatic lens was used to obtain the images at a 512 x 512 resolution. To avoid dye saturation, excitation power was set to 10% (488nm) and 25% (543nm) of maximum laser power. For all measurements an optical slice thickness of 10 µm was chosen.

Layer Height
Since the device simultaneously compresses six layers with the micrometer spindle, the layers need to have approximately the same height in order to apply equal strains to the layers. To verify this, height measurements were carried out. The initial idea was to measure the height using a micrometer head to slowly lower a glass tip onto a layer and look from the side with a bi-ocular to determine when the tip touched the layer. In this way the height could be read from the micrometer head. Because the edges of some of the layers curled up after a few days, they became higher than the centers of that layers and it was much more difficult to look from the side. This combined with the large distance between the sample and the bi-ocular, and the multiple layers of plastic of the well, disturbed the view too much to determine whether or not the tip touched the layer. The second option was to measure the height using the z-position of the confocal laser.
scanning microsoop. The CTG signal was used to focus the confocal laser scanning microsoop on the highest cells in the muscle layer and then focussed on the lowest cells. The difference of the z-position is taken as the height at that place. The height was measured at five different layers at two ages, 4 and 10 days. For each layer, this was done at nine different positions to determine the mean height of the layer.

An independent samples t-test was used to compare the mean height at both ages. One-way analyses of variances (ANOVA) were conducted, at both ages, to evaluate the relationship between the different layers and the height of the layer. The independent variable included five different layers. The dependent variable was the height of the layer. The ANOVA tests are assumed to be significant when $p < 0.05$. Follow-up tests were conducted to evaluate pairwise differences among the mean heights. When the variances were assumed to be equal, which was analyzed with a Leven’s test, a Least Significant Difference test is used to conduct post hoc comparisons, otherwise a Dunnett’s C test was used. The post hoc comparison tests are assumed to be significant when $p < 0.05$.

Cell Distribution
To compare the cell damage between multiple images of one layer and between different layers, it is necessary to know the cell distribution throughout the layers. To investigate this, five layers were stained with CTG and PI and 1000 µl of alcohol (70%) was added to the wells to induce cell death. Per well images were obtained at nine random chosen locations. At each of these nine locations images were taken at 20, 40, 60 and 80% of the height of the layer. This was done with the z-stack option of the CLSM, the auto-z-intensity correction compensated for fluorescent intensity variations at different heights in the layers.

A one-way ANOVA analysis of variance was conducted to evaluate the relationship between the height in the layers and the number of cells present. The height is the independent variable and the number of cells present in the images is the dependent variable. Follow-up tests were conducted to evaluate pairwise differences among the mean number of cells at a given height of 20, 40, 60, 80%. A Leven’s test was conducted to evaluate the variances and post hoc comparisons were carried out.

1.3 Compression Device
A compression device was developed to simultaneously compress six tissue engineered skeletal muscles. During compression of the layers the complete device was mounted on the CLSM, which gives the opportunity to acquire real time images. It contains six round indentors connected to a horizontal plate of stainless steel. The indentors consist of hollow plastic tubes with an outside diameter of 5 mm and a length of 40 mm and a small porous glass filter with a diameter of 3 mm and a height of 0.5 mm at the bottom of the tube, as shown in figure 1.1. The porosity of the filters is 10 µm. The indentors are able to move freely vertically through the plate and can individually be fixated with screws. The horizontal plate can be set to the desired position above the layers with three manipulators (x, y and z), with an accuracy of 100 µm. Furthermore, the device contains a fine micrometer head, with an accuracy 10 µm, for lowering the plate and thus applying the strain. The device further contains a heating plate for controlling the temperature of the culture medium. The device was aligned parallel to the culture well. This alignment was done using the CLSM in reflection mode. During lowering, the edges of the indentors were monitored to check whether they moved in the horizontal plane. If the device is aligned, no movement of the indentors is visible.
Figure 1.1 Schematic representation of experimental set-up used to compress the engineered skeletal muscle tissue
Chapter 2: Experimental setup

2.1 Protocol

Tissue engineered muscle layers (n=3) were subjected to 20% gross strain for a period of 8 hours. Furthermore, as a control, layers (n=3) only subjected to a minor strain due to the weight of the indentor and completely unstrained layers (n=3) were used. The experiments were carried out on 9-11 days old layers. The experiments were carried out with a 6-well culture dish containing layers, stained with CTG and PI, which was placed in the compression-device. The device was already mounted on the CLSM after being sterilized overnight with alcohol (70%). The height of the layers was measured. The horizontal plate was lowered with the z-manipulator such that the indentors did not touch the layers. To apply strain, the indentors were carefully lowered until they rested onto the layers and then secured with the fixation screws. The 20% strain was applied by lowering the horizontal plate using the fine micrometer head. At 80% of the height of the layer 10 images at random chosen positions under the indentor were taken. The layers were monitored by taking images at a half-hour interval for 4 hours and then with intervals of 2 hours for again 4 hours. During the experiment the culture wells were heated to remain a temperature of 37 degrees.

A one-way ANOVA analysis of variance was conducted to evaluate the relationship between the cell damage in the layers and the time. Follow-up tests were conducted to evaluate pairwise differences among the cell damage for the given timespan and to evaluate the differences between the three different compression groups. A Leven's test was conducted to evaluate the variances and post hoc comparisons were carried out.

2.2 Viability assay

To determine the cell viability in the constructs, the images taken with the confocal scanning microscop were analyzed using an image processing technique developed by Breuls et al, submitted. This method is based on a duo staining technique using CTG for detection of living cells and PI for dead cells. The amount of dead cells (nDC) is determined from the PI channel. In general, the amount of dead cells is easy to determine, since PI stains nuclei which appear as isolated circles in the PI image. However, occasionally these nuclei will overlap. Therefore, an improved version of previously used methods 8,9 has been implemented. The PI image is thresholded and a binary image is created of all pixel values above this threshold. The single nucleus areas were calculated thereby excluding cell debris and clusters of nuclei. Finally, the number of dead cells is computed by dividing the total area, covered by nuclei, by the mean single nucleus area. Thus the number of dead cells (nDC) is given by:

\[ n_{DC}(t) = \frac{\text{Total Nuclei Area}}{\text{Mean Single Nucleus Area}} \]  

The number of dead cells clearly depends on the number of cells present in a layer. Therefore, the number of dead cells alone cannot be used for comparing the amount of cell damage between layers with a different strain, because the total amount of cells in the image-plane is higher in a compressed layer than in an uncompressed layer. Therefore the amount of dead cells is normalized to the initial amount of cells present in the layer. This amount is determined using the CTG images at t=30 min.

First, the CTG image is thresholded, meaning that all pixel values below a certain threshold pixel value are set to zero. This step removes background noise. Second, the mean and standard deviation of the intensities in the thresholded image are determined. All pixel values that exceed the mean pixel intensity plus three times the standard deviation are set to this mean plus three times standard deviation pixel value. This step removes extremely bright pixels, which are caused by CTG accumulation. Third, the image obtained from the previous step is "stretched" and redistributed into 255 pixel values. This step basically normalizes the intensities of the image and compensates for intensity losses. Finally, the total intensity level of the image is determined by summation of the pixel values and divided by the image resolution (512 x 512). The amount of
living cells can be estimated from the total intensity levels, by multiplication with a scaling factor equal to the average intensity per living cell\(^7\).

This yields the number of living cells (\(n_{LC}(t=30)\)):

\[
n_{LC}(t = 30) = I_{CTG}(t) \cdot \frac{1}{c} \tag{2}
\]

in which \(I_{CTG}(t)\) is the total CTG intensity level in an image taken at time \(t\) and \(c\) the scaling factor (\(c=3800\)).

The amount of damage (%) is calculated using equation (3).

\[
Damage(t) = \left( \frac{n_{DC}(t)}{n_{LC}(t = 30)} \right) \cdot 100\% \tag{3}
\]
Chapter 3: Results

3.1 Tissue Engineered Skeletal Muscle

The engineered skeletal muscle layers were disc-shaped and had a diameter of approximately 15 mm. 60% of the muscle cells were differentiated from myoblasts into myotubes. All layers contained regions of myotubes that were aligned. However, the directions of these alignments varied throughout the layers. The muscle layers shrank during the first 8-10 days, due to the interaction of the muscle cells and the collagen and Matrigel. After 8 days, some myotubes showed spontaneous contractions, demonstrating differentiation of the cells. The layers could be kept under these conditions for at least 30 days.

Some muscle layers could not be used for compression experiments, due to two causes. First, air bubbles get trapped inside the layers during making of the muscle layers. These air bubbles all disappeared in the first days, but when they were too big the muscle cells had settled around them. This caused irregularities within the structure of the layer and therefore these layers could not be used. Second, sometimes the cells at the edge of the muscle layers were not strong enough to withstand the internal forces and the edges curled up, causing the layers to detach from the wells.

3.2 Characterization of the layers

Layer height

![Figure 3.1. Heights of five different four and ten day old muscle layers](image)

Figure 3.1 shows the heights of five layers (identified with A, B, C, D and E) of four and ten days. The height of the four days old layers was $748.33 \pm 48.17 \mu m$ (mean \pm SD). The height of the ten days old layers was $489.44 \pm 57.27$ (mean \pm SD), which was significantly ($p < 0.001$) lower for all five layers. The one way ANOVA was significant for the four day old layers. The results of the follow up test (Least Significant Difference), as well as the means and standard deviations for the five layers, are reported in table 3.1. There was no significant difference between heights of the five layers, except for layer D, which was higher than the others.

<table>
<thead>
<tr>
<th>Layer</th>
<th>M</th>
<th>SD</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>720.33</td>
<td>30.545</td>
<td>NS</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>754.56</td>
<td>47.556</td>
<td>NS</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>733.22</td>
<td>50.428</td>
<td>NS</td>
<td>NS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>802.22</td>
<td>30.605</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td></td>
</tr>
<tr>
<td>E</td>
<td>731.33</td>
<td>36.218</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>*</td>
</tr>
</tbody>
</table>

Note: NS = Nonsignificant differences between pairs of means, while an asterisk (*) denotes significance using the LSD procedure.
For the ten day old layers the one way ANOVA was not significant. Thus at this age the measured height was not significantly influenced by the layer and therefore layers of this age are used for the compression experiments. Table 3.2 shows the means and standard deviations of the ten day old layers.

Table 3.2. Means and standard deviations of height from ten day old layers

<table>
<thead>
<tr>
<th>Layer</th>
<th>M</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>515.11</td>
<td>29.747</td>
</tr>
<tr>
<td>B</td>
<td>486.56</td>
<td>97.234</td>
</tr>
<tr>
<td>C</td>
<td>476.22</td>
<td>58.611</td>
</tr>
<tr>
<td>D</td>
<td>511.11</td>
<td>33.005</td>
</tr>
<tr>
<td>E</td>
<td>458.22</td>
<td>24.616</td>
</tr>
</tbody>
</table>

Cell Distribution

Figure 3.2 shows the distribution of cells at four different heights. The one way ANOVA was significant. The results of the Dunnett’s C tests, as well as the means and standard deviations for the four heights, are reported in table 3.3. There were significant differences between the means for all the heights except for the 40% and 60% height. The 80% height group showed the highest number of cells.

Table 3.3. Differences in number of cells, measured at different heights in the layers.

<table>
<thead>
<tr>
<th>Height</th>
<th>M</th>
<th>SD</th>
<th>20%</th>
<th>40%</th>
<th>60%</th>
</tr>
</thead>
<tbody>
<tr>
<td>20%</td>
<td>194.99</td>
<td>76.542</td>
<td>*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>40%</td>
<td>257.46</td>
<td>101.287</td>
<td>*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>60%</td>
<td>317.57</td>
<td>126.176</td>
<td>*</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>80%</td>
<td>437.94</td>
<td>173.047</td>
<td>*</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Note: NS = Nonsignificant differences between pairs of means, while an asterisk (*) denotes significance using the Dunnett’s C procedure.

3.2 Compression experiment

In the compression experiment three layers would be strained and two control groups of three layers would be strained. Due to detaching of the layers and irregularities within them, some layers could not be used for the experiment. Therefore the number of layers within the control group with only the indenter resting on the layer was lowered to just one layer and the unstrained control group was lowered to two layers.
In Figure 3.3 an image taken with the confocal laser scanning microscope can be seen. This image was taken precisely under the edge of the indentor during straining, it clearly shows that the strain applied causes a local cell damage under the indentor and that the rest of the cells stay alive.

![Confocal laser scanning microscope image](image)

Figure 3.3. Confocal laser scanning microscope image half under the indentor, indicating local cell damage (red nuclei) under the indentor and viable cells (green) next to the indentor.

Figure 3.4 shows the cell normalized cell damage in ten day old muscle layers (n=3) with 20% strain, unstrained muscle layers (n=2) and muscle layers with only the porous indentor resting on them (n=1). A Dunnett’s C test showed that for the complete time span the total percentage of cell damage was significantly higher in 20% strained layers than in the control groups. The cell damage in all three groups increases at t = 480 min. Cell damage in the 20% strained layers increased significantly from 27.7% (30 min) to 76.2% (360 min), whereas cell damage in the unstrained controls remained constant over time with an average value of 4.32% ± 7.31%. The control layers with the indentors resting on them show an initial cell damage (17.83%) which remains constant with an average value of 17.83% ± 6.81%. Table 3.4 summarizes normalized cell damage in the three different compression states, calculated as a percentage of the total number of cells present at t = 30 min.
Table 3.4. Normalized cell damage (%) at strains 0 and 20 %.

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>0%</th>
<th>0% with indentor</th>
<th>20%</th>
</tr>
</thead>
<tbody>
<tr>
<td>30</td>
<td>0.8</td>
<td>15.0</td>
<td>27.7</td>
</tr>
<tr>
<td>60</td>
<td>2.2</td>
<td>17.8</td>
<td>42.6</td>
</tr>
<tr>
<td>90</td>
<td>2.8</td>
<td>12.7</td>
<td>57.9</td>
</tr>
<tr>
<td>120</td>
<td>4.0</td>
<td>17.1</td>
<td>71.8</td>
</tr>
<tr>
<td>150</td>
<td>4.2</td>
<td>17.0</td>
<td>68.0</td>
</tr>
<tr>
<td>180</td>
<td>3.7</td>
<td>18.1</td>
<td>75.7</td>
</tr>
<tr>
<td>210</td>
<td>9.1</td>
<td>21.1</td>
<td>83.1</td>
</tr>
<tr>
<td>240</td>
<td>3.9</td>
<td>21.1</td>
<td>71.3</td>
</tr>
<tr>
<td>360</td>
<td>9.3</td>
<td>20.7</td>
<td>76.2</td>
</tr>
<tr>
<td>480</td>
<td>5.8</td>
<td>38.1</td>
<td>94.3</td>
</tr>
</tbody>
</table>
Discussion

Pressure sores are localized areas of degenerated skin and underlying soft tissues caused by sustained mechanical loads and often starting in the muscle tissue. There is much debate on the etiology of the pressure sores. Therefore an experimental model system and a compression device were developed which enable to study the hypothesis that cell deformations directly lead to cell damage in muscle tissue. In this study the newly developed model system was characterized and the consequences of sustained cell deformation in terms of local cell damage were studied.

The model system contains engineered skeletal muscle layers and it is an improved representation of the in-vivo muscle tissue. The protocol resulted in reproducible layers that have the same height and cell distribution. The compression device can be used to simultaneously compress six muscle layers. The muscle cells of uncompressed layers stay alive for seven hours approximately and then the cells dye due to rising of the pH. When 20% strain is applied to tissue engineered muscle layers, cell damage significantly increases compared to the control groups. Since predisposing factors for cell damage, such as oxygen and nutrient supply to the layers were equal for strained and unstrained layers, the deformation of the layers and associated cells was considered as the trigger for cell damage.

The model system is an improved representation of in-vivo muscle tissue compared to current models (e.g. Bouten et al, 1999). The cells are imbedded in a mixture of collagen I and Matrigel, instead of in agarose. This mixture is a better representation of the in-vivo surroundings of muscle cells, the cells are not immobilized and are able to differentiate. The model system does not consist of nerves or blood vessels, which can be seen as a lack in the representation of the in-vivo muscle. But for studying the hypothesis that cell deformation leads to cell damage in stead of the occlusion of blood vessels, it is rather fine that model doesn’t contain blood vessels, because the cell damage of the compression experiment cannot be caused by occlusion of them.

However, there are a few discussion points. First, not all the cells were aligned, which is obvious the case in an in-vivo muscle. Second, some layers could not be used because of irregularities in the internal structure and detachment of the muscle layers. Therefore, it was not possible to compress six layers as the compression device was designed for. Changing the concentration of the muscle cells in the layers could solve this problem. A remark has to be made about the cell distribution in z-direction. This distribution was not homogeneous throughout the layers, the cells were migrated to the top of the layers. This is no problem when you measure at the same height in all layers, because at one height the cells are homogeneously distributed and it is possible to compare the cell damage. But when different heights are compared the initial cell amount has to be taken in to account.

When studying the influence of strain on cell damage, ideally all the cell damage measured has to be caused by the applied strain. In the compression experiment the lowering of the indentors of the compression device onto the muscle layers initiated an initial cell damage. This initial cell damage cannot be distinguished from the cell damage due to the applied strain and therefore has to minimized. Improving the lowering mechanism of the indentors will do this.

To extend the life span of the cells in the compression device from seven till twenty four hours, the device will be extended with an incubation system for controlling the CO2 level and the humidity around the muscle layers. Measuring the height of the layer was done with a CLSM, it is known that the z-resolution of such a microscope is not very good. Therefore the results of the height of the muscle layers are likely to be too high, approximately 2-2.5 times to high (data not shown). This implies that the applied strain in the compression experiment probably was not the 20% strain as mentioned, but 40-50%. To compare different strains this height measurement has to be improved. A possibility is to attach force indicators to the indentors, this way the height of the layers could be measured much better.

In conclusion, a model system and compression device which enables the study the hypothesis that cell deformations directly lead to cell damage in muscle tissue. It is suitable for studying effects of sustained cell deformation over time. At 20% strain the cell damage was clearly higher then in the control groups, indicating that cell damage related to pressure sores within a tissue engineered muscle layer can be induced and that this cell damage can be quantified using a viability assay.
References

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Appendix 1: Matlab model

% viabilitycompression.m
% Imageprocessing Confocal Images: Dammage Assessment Compression Experiment
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%
% This program loads confocal images and determines the number of living
and dead cells.
% 
% Input:
% 
% Image files with the following file format:
% 
% s0-t30-A1.jpg
% 
% s0 = amount of strain
% t30 = time
% A1 = layer ID + position
% 
% Output:
% 
% The matrix "data" containing all the information
% 
% column 1 = strain
% column 2 = time
% column 3 = layerid
% column 4 = position
% column 5 = number of cells

% Before you begin, you have to define a few things:
% 
% 1. How much strain is applied in percentage
% 2. The thresholds of the PI and CTG channel
% 3. The minimum size of the nuclei
% 4. How the layers are called.
% 5. Switch on the desired channels

% This can be done in the define variables section

% Clearing memory and screen. Closing all the open figures.

clear; clc; format compact;
close all;

% Initialize counter and data matrix
counter=1; tel=1;
data=[];
mean_area=[];

% Define variables

strain = 20 % Define strain (%)
thPI = 150; % Define threshold PI
minsize = 15; % Define minimum size of the nuclei
layerid = {'A', 'B', 'C', 'D', 'E', 'F'}; % Define layerid's
timeseries = [30 60 90 120 150 180 210 240 360 480]; % Define timeseries

% Choose which channels you want to use (0 = off; 1 = on)
CTGchannel = 1 % Switch CTG Channel on/off
PIchannel = 0 % Switch PI Channel on/off

if strain==0
cd ('d:\my documents\my pictures\rolf\compressionexperimentstrain0')
elseif strain==10
cd ('d:\my documents\my pictures\rolf\compressionexperimentstrain10')
elseif strain==20
cd ('d:\my documents\my pictures\rolf\compressionexperimentstrain20')
elseif strain=='0tip'
cd ('d:\my documents\my pictures\rolf\compressionexperimentstrain0tip')
end

%Read Image(s)
for time=1:10 %length(timeseries)
  for layer=4:4
    for pos=1:20
      if strain =='0tip'
        name=['stip0-t',num2str(timeseries(1,time)),'-',
             'con',num2str(pos),'.tif']
      else
        name=['s',num2str(strain),'-t',num2str(timeseries(1,time)),
             '-', num2str(layerid(1,layer)),num2str(pos),'.tif']
      end
      if strain == '0tip'
        data(counter,1)=0;
      else
        data(counter,1)=strain;
      end
      data(counter,2)=timeseries(1,time);
      data(counter,3)=layer;
      data(counter,4)=pos;

      % Read image
      im=imread(name,'tif');

      if CTGchannel == 1
        % Extract CTG Channel
        g=im(:,:,2);
        g=im2double(g);

        % figure;imhist(g);
        % figure;imshow(g);title(name);

        % Determination threshold
        imh=imhist(g);
        imh=imh(5:250);
        q=find(imh==max(imh))+5;
        thCTG=q(1)
% Thresholding
k=im2bw(g,thCTG/255);
% figure; imshow(k)
k=im2double(k);
g=im2double(g);
g=g.*k;
% figure;imshow(g);title('Thresholded image');

delete all zero entries in g
[i,j,s] = find(g);

% determine mean and standard deviation of all values above threshold
gmean=mean(s);
gstd=std(s);

% Determine upper threshold
upth=gmean+3*gstd

% All values above upth will become upth
k=upth.*(g>upth);
m=g.*(g<upth);
g=k+m;
% figure; imshow(g);title('Peak values cut off');

% Stretchen van image
if upth>1
    upth=1;
end

g=g.*1/(upth);
% figure;imshow(g);title(name);
Intensitylevel(tel)=sum(sum(g))/(size(g,1)*size(g,2));

CTG(tel)=Intensitylevel(tel).*(1/4.74E-4);
end

if PIchannel == 1

%Extract Red Channel
r=im(:,:,1);
% figure;imshow(r);title(name);

% Thresholding & make binary image
k=im2bw(r,thPI/255);
r=im2double(k);

% figure;imshow(r);title('Thresholded image');

% Apply "erode" operation
SE=ones(2,1);
c=imerode(r,SE);
% figure; imshow(c); title('eroded image SE=2');

% Apply "clean" operation
t=bwmorph(c,'clean');
% figure; imshow(c); title('Cleaned image');
Area=[]; stdmean_area=0; f=[]; X=[]; maxarea=0;

% Count number of nuclei
[X, num] = bwlabel(t);
X = sparse(X);
Q = zeros(size(X));
for n = 1:num
    Area(n) = full(sum(sum(X == n)));
    Q = Q + Area(n) .* (X == n);
end

f = Q > minsize;
Area = Area .* (Area > minsize);
Area = Area .* (Area < 65);
[i, j, s] = find(Area);
mean_area(counter) = mean(s);

% figure; imshow(f)
if mean_area(counter) > 0
    PI(counter) = sum(sum(f)) / mean_area(counter);
else
    PI(counter) = 0;
end

data(counter, 5) = PI(counter);
end

% Raise counters
tel = tel + 1;
counter = counter + 1;
end
end
if PIchannel == 1
    data
    mean_area = mean_area';
    save dataB10nuclei65.dat data -ASCII
    save dataB10mean_areaNuclei65.dat mean_area -ASCII
end
if CTGchannel == 1
    save datastrain10layerD_t30_CTG.dat CTG -ASCII
end