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# Cell death induced by mechanical compression on engineered muscle results from a gradual physiological mechanism

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ABSTRACT

Deep tissue injury (DTI), a type of pressure ulcer, arises in the muscle layers adjacent to bony prominences due to sustained mechanical loading. DTI presents a serious problem in the clinic, as it is often not visible until reaching an advanced stage. One of the causes can be direct mechanical deformation of the muscle tissue and cell. The mechanism of cell death induced by mechanical compression was studied using bio-artificial skeletal muscle tissues. Compression was applied by placing weights on top of the constructs. The morphological changes of the cytoskeleton and the phosphorylation of mitogen-activated protein kinases (MAPK) under compression were investigated. Moreover, inhibitors for each of the three major MAPK groups, p38, ERK, and JNK, were applied separately to look at their roles in the compression caused apoptosis and necrosis. The present study for the first time showed that direct mechanical compression activates MAPK phosphorylation. Compression also leads to a gradual destruction of the cytoskeleton. The percentage apoptosis is strongly reduced by p38 and JNK inhibitors down to the level of the unloaded group. This phenomenon could be observed up to 24 h after initiation of compression. Therefore, cell death in bio-artificial muscle tissue caused by mechanical compression is primarily caused by a physiological mechanism, rather than through a physical mechanism which kills the cell directly. These findings reveal insight of muscle cell death under mechanical compression. Moreover, the result indicates a potential clinical solution to prevent DTI by pre-treating with p38 or/and JNK inhibitors.


## 1. Introduction

A pressure ulcer (PU) is an injury in skin and underlying tissues, mainly caused by too much and/or too long compression imposed on the tissue (National Pressure Ulcer Advisory Panel, 2014). Particular patients with impaired sensation or mobility are vulnerable to developing PUs (Byrne and Salzberg, 1996; Garber and Rintala, 2003). Among PUs, deep tissue injury (DTI) is a very serious problem in clinical treatments, as it is often not visible until reaching an advanced stage (Bliss, 1998; Bouten et al., 2003). DTI may originate in skeletal muscle tissue, which has been shown to be particularly susceptible to damage development as a result of tissue compression (Daniel et al., 1981; Nola and Vistnes, 1980). Direct mechanical compression, ischemia, lymphatic blockage and reperfusion are potential factors triggering the damage development of muscle tissue (Dinsdale, 1974; Kosiak, 1959; Miller and Seale, 1981). Furthermore, it was reported that these factors may contribute to different stages of the damage (Bouten et al., 2003; Gawlitta et al., 2007b; Stekelenburg, 2005).

Direct mechanical deformation of the tissue and cell is considered to be one of the major triggers of DTI, and the importance of its role in compression induced tissue damage has recently been emphasized (Loerakker et al., 2011). An in-vivo study by Stekelenburg (2005) showed that, after applying compression to rat skeletal muscle, the areas of tissue damage correlated well with the regions that underwent most deformation. Similar results (Gawlitta et al., 2007a) were reported in an in vitro study on bio-artificial muscle tissues, which demonstrated that direct mechanical deformation itself is sufficient to invoke evident damage within 24 h. All these findings support the hypothesis that mechanical compression is a very important factor in the initialization of cell damage. However, the specific etiological pathways involved remain poorly understood.

Considering the potential sensor for mechanical compression, one promising candidate could be cytoskeletons. Actin filaments support the fragile plasma membrane and provide the mechanical linkage that lets the cell sense and respond to the strains (Janmey and Weitz, 2004). Actin cross-linking protein, α-actinin, could also be a mechanical sensor participating in cell death. Since the

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compression causing pressure ulcers is mostly applied perpendicularly to the length of muscle fibers, a 'damaging' track coincides with the distribution of the sarcomeric α-actinin could happen directly. Furthermore, the actinins were recently reported to modulate the function of mechanical sensors at the membrane and at the Z-line, followed by the activation of the downstream targets involving cell viability (Lek and North, 2010).

Direct mechanical compression might also be converted to biochemical signaling pathways. Mitogen-activated protein kinase (MAPK) cascades are capable of transmitting mechanical stimuli to the nucleus, regulating cell functioning and viability (Widmann et al., 1999). The MAPK family mainly consists of three subfamilies: p38 MAPK, extracellular regulated kinase 1 and 2 (ERK1/2) and Jun $NH_2$-terminal kinase (JNK). In skeletal muscle, cell viability has been connected to the phosphorylation (activation) of MAPK when induced by mechanical loading (Burkholder, 2007; Carlson et al., 2001; Martineau and Gardiner, 2001). However, the majority of the reports focus on mechanical loading under stretch or muscular overloading than on mechanical compression.

Therefore, in this study the influence of direct mechanical compression of the bio-artificial muscle tissues on MAPK activation, cytoskeleton (expression and distribution), and cell death (apoptosis and necrosis) was investigated (Gawlitta et al., 2007a). In addition, the contribution of MAPK activities to the development of apoptosis and necrosis under direct mechanical compression was studied. The hypothesis is that direct mechanical deformation imposed on skeletal muscle induces the phosphorylation of some or all of the three MAPKs: p38, ERK and JNK. Activating some or all of these MAPK pathways will trigger both cell death activation and protection mechanisms contributing to a gradual increase in cell death. Meanwhile, the direct mechanical deformation also disturbs the original organization of the cytoskeleton and finally leads to cell death.

## 2. Material and methods

Tissue engineered muscle was created by suspension of C2C12 murine skeletal myoblasts (passages 10–15, ECACC, Salisbury, UK) in a rat-tail collagen I (3.2 mg/ml, BD Biosciences)/matrigel (BD Biosciences) mixture (Breuls et al., 2003; Vandenburgh et al., 1996). The constructs were covered with growth medium for 24 h. Afterwards, the growth medium was replaced by differentiation medium (DM). The engineered muscle constructs were allowed to differentiate and mature for 7 days until the experiments were performed.

Compression induced activations of p38, ERK and JNK were tested by Western blotting for eight different periods of compression: 5 min, 10 min, 30 min, 60 min, 2 h, 6 h, 14 h, and 24 h. Individual experiments were repeated three times.

For measuring the expression alterations of α-actinin and actin, Western blotting was done on samples after the same eight different periods of compression. For looking at the changes of cytoskeleton structure, histological studies showing the distributions of actin and α-actinin were conducted after a selection of three different time of compression: 5 min, 2 h, and 24 h.

Apoptosis and necrosis activated by compression was assessed using confocal microscopy, which gave real time information for 24 h. Inhibitors of p38, ERK and JNK were used separately to block the activity of the particular MAPK pathway, and the cell death by compression was investigated (Cuenda, 1999; Kim and Lee, 2009).

The experimental group was treated with unconfined tissue compression (+C). The mean undeformed height of the samples was determined as $530 \pm 69$ μm ($n=16$). A tissue compression of $34 \pm 8$% was achieved by placing rectangular stainless steel weights on top of the constructs on day S7, with spacers of 350 μm at their four corners (Gawlitta et al., 2007b). The chosen value of compression was based on a previous finding that compressive strains of 20% and 40% are able to cause development of muscle damage (Gawlitta et al., 2007a) and that clinical evidences show that strains up to 50% are possible by sitting or lying on a hard surface, or as a result of medical devices, folds in clothing, or repositioning (Linder-Ganz et al., 2007; Ohura et al., 2008; Oomens et al., 2013). The control group did not get compressed (−C) while all the other conditions stay the same as the experimental group.

SB 203580 (SB) (S8307, Sigma-Aldrich) is an inhibitor of the activity of phosphorylated p38 (Cuenda et al., 1995), while PD 098059 (PD) (P215, Sigma-Aldrich) specifically inhibits the ERK signaling pathway at the point of MEK (Alessi et al., 1995) and SP 600125 (SP) (S5567, Sigma-Aldrich) acts as a selective and reversible inhibitor of JNK (Bennett et al., 2001). In case of using one of these inhibitors in the experiment, bio-artificial muscles were pretreated in DM with or without 10 μM SB, 24 μM PD, or 20 μM SP one hour before the experiment.

At the end of the compression, the region of bio-artificial muscles under compression was cut off along the two parallel lines of the indenter. Protein lysate was prepared by ultra-sonication. The lysate was then centrifuged (8000*g* at 4 °C for 10 min) and the supernatant was collected. Electrophoresis was conducted on 10% or 12% SDS-polyacrylamide gels and transferred onto polyvinylidene fluoride (PVDF) membrane (Immobilon-P Membrane, Millipore). Membranes were incubated at 4 °C overnight with the primary antibody in TBST containing 5% milk or BSA. The membranes were then incubated for 2 h with respective secondary antibody. Subsequently, the signals of the bands were developed by using the ECL detection kit (34075, Thermo Scientific) and visualized using the Versa-doc 3000 (Bio-Rad) system.

To prepare bio-artificial muscles for cryosectioning, they were fixed for 1 h in 10% formalin (Sigma Aldrich), snap-frozen in Tissue-Tek OCT compound (Sakura Finetek Europe B.V.) using ice-cold 2-methylbutane (Sigma Aldrich), and sectioned into 10 μm slices.

For immunocytochemistry staining, samples were permeabilized with 1% Triton-X-100 (Merck) in PBS for 20 min. Afterwards, they were incubated for 2 h with antibodies against sarcomeric α-actinin (1:800) (clone EA-53; Sigma-Aldrich). Next, sections were incubated for one hour with Alexa Fluor 555-conjugated goat anti-mouse IgG1 (1:200) secondary antibodies (A21127; Invitrogen) and phalloidin (for actin). Finally, they were stained for an additional 5 min with DAPI (0.1 μg/ml) (Sigma Aldrich). Sections were evaluated using fluorescence microscopy (Axiovert 200M, Zeiss).

DM with 8 μM PI (P3566, Invitrogen BV) and 1 μM YP (Y3603, Invitrogen BV) was added to the sample one hour before scanning. The progression of cell death was then monitored on a confocal laser-scanning microscope (Axiovert 100M, Zeiss or LSM 510M, Zeiss). The sample was placed in a chamber box with conditions of 37 °C and 5% $CO_2$, and the compression was applied for 24 h. At the beginning of every hour, 12 images were sequentially scanned (pinhole=200) from the bottom to the top of the sample resulting in a Z-stack with total thickness of 120 μm.

All data are presented as mean ± standard error of the mean or the mean only. Differences over time and between groups were assessed using an analysis of variance (ANOVA) performed with Prism software, version 5.01. A Bonferroni post-hoc test was then applied and statistical significance was prescribed at the 5% level ($^{*}p<0.05$; $^{**}p<0.01$; $^{***}p<0.001$).

## 3. Results

Under compression, p38, ERK and JNK showed different patterns of activation in time. The expression of phosphorylated p38 (p-p38), the activated form of p38, was elevated after 5 and 10 min of compression. After 20 min p-p38 expression returned to the control level. However, at 24 h the p-p38 expression increased again (Fig. 1(1)). The total content of p38 (p38) comprising of phosphorylated p38 and unphosphorylated p38 was not affected by compression during the first 6 h. In contrast, after 14 and 24 h a significant reduction of p38 expression was found in the compression group when compared to control samples (Fig. 1(2)).

There are two types of ERK: the 44 kDa ERK1/p44 and 42 kDa ERK2/p42, resulting in two phosphorylated forms: phosphorylated ERK1/p44 (p-ERK/p44) and phosphorylated ERK2/p42 (p-ERK/p42). Both p-ERK/p44 and p-ERK/p42 were activated in a similar way by compression. 5 min and 10 min compression increased their expression, while in the 20 min to 6 h groups p-ERK/p44 and p-ERK/p42 expression returned to the basal level. However, after 14 h compression, the expression of both p-ERK/p44 and p-ERK/p42 was significantly reduced (Fig. 2(1) and (2)). Moreover, there was no significant change of total content ERK expression until 14 h of compression, after which a reduced expression was observed (Fig. 2(3) and (4)).

The activated form of JNK, phosphorylated JNK (p-JNK) was detected in the compressed groups only after 5 min and 10 min compression. For all the other groups of p-JNK, no or very weak signals were detected (Fig. 3(1)). The expression of total JNK remained unchanged throughout the experimental time (Fig. 3(2)).

The expression level of α-actinin was not changed throughout the 24 h compression and there was no significant difference