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Effects of uniaxial stretching on tenocyte migration behaviour

https://doi.org/10.1515/cdbme-2018-0076

Abstract: It is widely known that tendon tissues are subjected to repeated cyclic mechanical load which influences cellular processes. The involvement of principles of mechanics in tissue engineering contributes to the investigations of the connection between mechanical and biological parameters in cellular processes and as well as to the development of new approaches for specific treatment methods. The healing process of injured tendons includes tenocyte migration which occurs from intact regions of tendon into the wound site. The aim of the present study is to investigate and enhance the migration characteristics of tenocytes under uniaxial mechanical stretching using an in-house tensile bioreactor system. Uniaxial mechanical stretching is applied to tenocyte-seeded silicone as well as collagen membranes, which possess different material properties. Tenocyte-seeded silicone membranes were investigated under three different loading conditions, including unstimulated (control), 3% and 5% strain, at frequency of 0.5 Hz. Tenocyte-seeded collagen membranes were investigated using three different frequencies, including unstimulated (control), 0.1 Hz and 0.5 Hz at strain of 4%. The main finding in this study is that uniaxially mechanical stretching at 3% strain enhances the cell migration more than 5% strain on silicone membranes.

Keywords: Tendon Tissue Engineering, Uniaxial Stretching, Tenocytes, Silicone Membrane, Collagen Membrane

1 Introduction

Current therapeutic options are often inefficient for tendon ruptures whose incidence has increased dramatically in the past 50 years [1-2]. Therefore, there is a huge need for promising methods in order to facilitate the tendon healing process which occurs slowly and results in lack of structural integrity and mechanical strength of tendon tissue [2-3]. Since tendon tissue is subjected to mechanical loads, a better understanding of the connection between the mechanical and biological parameters in tenocyte cellular mechanisms might provide new approaches for advanced treatment methods [4]. Tendon tissue engineering therefore utilizes mechanical cell stretching which has been introduced an attractive signal to create mechanically functional tissues while promoting beneficial cell responses [5]. Previous studies have been shown that mechanical stretching induces the regulation processes of tendon cells such as collagen synthesis and repair activity [6-7]. Nonetheless, little is known about the effects of mechanical stretching on tenocytes migration behaviour which plays a crucial role in tendon healing [8].

In this study, tenocytes were mechanically stimulated to test the hypothesis that the migration characteristics of tenocytes can be enhanced by uniaxial mechanical stretching. Enhancement of tenocyte migration is intended to speed up the tendon healing process by faster movement to defect site and production of extracellular matrix proteins which increase mechanical strength of tendon tissue. By this motivation, uniaxial mechanical stretching is applied to tenocyte-seeded silicone as well as collagen membranes through use of an in-house tensile bioreactor. The in-house tensile bioreactor enables to apply different strain, frequency, strain rate and insertion of rest periods which have been found to influence important cell regulatory effects[5]. Therefore, we have applied different mechanical stimulation parameters to find the appropriate one for the enhancement of tenocytes migration. Since a homogenous distribution of normal stresses in the specimen is significant for tensile test experiments, the membranes were cut into the dogbone shape.

2 Materials and Methods

2.1 Isolation of Tenocytes

Tenocytes were obtained under sterile conditions from the mice Achilles tendon. Tendon tissue was cut into small pieces and each piece was placed into the well of 6-well cell culture plates. Dulbecco’s modified Eagle’s medium (DMEM) or DMEM-F12 containing 50% (v/v), fetal bovine serum (FBS), 1% (v/v) penicillin/streptomycin was added to each well. The composition of medium was kept the same until the tenocytes migrated out from the tissues. Following the migration of tenocytes into the wells, the FBS proportion was reduced gradually
to 10%(v/v). After cell expansion in in vitro conditions, the tenocytes were seeded on dog-bone shaped carrier materials with the cell number of 120.000 cells/cm². The cells of passages 2–4 were used for the following experiments.

2.2 Mechanical Characterization of Carrier Materials

Silicon membranes made of poly(dimethylsiloxane) (PDMS) and collagen membranes consisting of 120 mg/ml collagen type 1 (kindly provided from Amedrix GmbH, Esslingen, Germany) were used as a carrier material for cell seeding. Before cell seeding and mechanical stretching, silicone and collagen membranes were mechanically characterised by performing stress-relaxation tests. Stress-relaxation tests are performed by using an in-house tensile bioreactor. Membranes were cut in a dog-bone shape form and submerged in a phosphate-buffered saline (PBS) solution to mimic physiological conditions. For stress-relaxation tests, they were strained with a rate of 0.05 m/s and held at constant strain for 5 minutes. The reaction force was measured over time.

2.3 Uniaxial Mechanical Stimulation in a Tensile Bioreactor

Following five days of incubation of tenocyte-seeded silicone membranes, they were exposed to uniaxial strain of 3% and 5 % at frequency of 0.1 Hz (n=3 for each strain). Each membrane was subjected to tensile strain four times for 15 minutes every day (in total of 5 days). Every 15 minutes stimulation was followed by an hour-long pause. Furthermore, tenocyte-seeded collagen membranes were loaded with a strain of 4% using two different frequencies: 0.5 Hz and 0.1 Hz (n=1 for each frequency). Duration and period of mechanical stimulation was identical with silicone membranes.

2.4 Cell Viability Assay

Live and dead tenocytes were visualized with Calcein AM and Propidium Iodium (PI), respectively. The pieces taken from clamp area of carrier materials were washed in PBS at room temperature, followed by incubation with 10 µM Calcein AM for an hour. After washing of specimens in PBS, they were incubated with 25 µM PI for 10 minutes, and then washed again in PBS. Fluorescence images were taken by using an inverted fluorescence phase contrast microscope (Keyence BZ 8100, Japan), and cell viability was quantified in images from three biological replicates, using an ImageJ script. Cell viability is calculated in percentage of living to total cells.

2.5 Cell Migration Assay

The cell migration assay was conducted using transwell inserts with pore size of 8 micron. For this migration assay, statically cultured and stretched cells were trypsinized from the membranes and resuspended with 300 µl of serum-free medium and seeded on the upper chamber of transwell membranes. The lower chamber is filled with 600 µl of DMEM with 10% FBS as a chemo-attractant. The tenocytes were incubated in 5% CO₂ at 37°C for 3 h. Subsequently, the tenocytes on the upper membranes were removed using a cotton swab. The tenocytes, which migrated to the bottom of the membrane, were fixed in 10% formalin for 15 min and stained using 300 nM DAPI for 5 min. The tenocytes on the bottom of the membrane were counted using an ImageJ script to evaluate the numbers of migrated cells.

3 Results

3.1 Mechanical Characterization of Carrier Materials

The reaction force is plotted over time (see Figure 1). The reaction force of collagen membrane decreased over time at constant strain. For silicone membranes, measured force was constant during constant strain. Thus, it has been observed that collagen membrane shows a viscoelastic behaviour and it may be well-suited as a replacement material for tendon repair. In contrast, silicone membranes showed a purely elastic behaviour as expected.

![Fig. 1: Reaction forces over time.](image-url)
3.2 Uniaxial Mechanical Stimulation in a Tensile Bioreactor

The tensile bioreactor introduced in this study provides real-time information on the displacements and forces applied to the carrier materials throughout the duration of the culture period [9]. The bioreactor system (see Figure 2) enables the dynamic cyclic stretching of tenocytes and supports long term cell cultivation in a climate controlled environment. Representative graphs of real-time displacement and force during mechanical stretching can be seen in Figure 2.

Fig. 2: Upper- A tenocyte-seeded membrane is stretched uniaxially in a custom-made tensile bioreactor. Lower- Displacement and force graphs.

3.3 Cell Viability Assay

The cell viability assay has been performed in order to examine whether transmitting external forces into the cell has an effect on cell viability. The percentage of cell viability was lower for the unstimulated control group than for the silicone membranes stretched with 3% strain. Conversely, cell viability was higher on the control group than the silicone membranes stretched with 5%. In Figure 3, it is shown that the percentage of live cells on 3% stretched membranes is higher than on 5% stretched membranes. Representative pictures of fluorescence microscopy for the stained cells can be seen on Figure 4. For the tenocytes seeded on collagen membranes, the percentage of cell viability was highest for the control group in comparison to stretched membranes (results are not shown).

Fig. 3: Comparison of cell viability of 3%, 5% stretched membrane and control groups.

Fig. 4: Fluorescence microscopy images (green-live cells, red-dead cells) of cells on the silicone membrane after mechanical stretching. Left-3% of strain. Right-5% of strain.

3.4 Cell Migration Assay

Tenocyte migration plays a critical role in tendon healing. A transwell cell migration assay was performed in order to evaluate the effect of mechanical stretching on the migration of tenocytes. As shown in Figure 5 on the graph, mechanically stretched cells on silicone membranes migrated faster than those of the control groups. The 3% strain enhanced greater cell migration than the 5% strain (p=0.031<0.5). There was no significant difference between the cell migration behaviour of control and 4% stretched collagen membranes (results are not shown).

Fig. 5: Transwell migration assay results of 3%, 5% stretched membrane and control groups.
4 Discussion

Cellular processes of tendon tissue are affected by mechanical loading. Therefore, the investigations, which address the effects of mechanical loading, are important in order to gain a better understanding of tendon mechanobiology. Cell migration is one of the essential process for tendon damage repair. In previous studies, it has been demonstrated that the cell migration can be promoted by various stimulus, such as laser phototherapy, the use of rat bone marrow mesenchymal stem cell-derived conditioned medium and mechano-growth factor (MGF) [10-11-12]. However, the present study provides new proof that mechanical stretching might be used to accelerate tendon repair by promoting the migration of tenocytes.

The aim of present study was to investigate the effect of mechanical stretching on the cell viability and the cell migration of tenocytes. With this aim, cell viability and cell migration assays were carried out for tenocyte-seeded and mechanically stimulated or unstimulated silicone and as well as collagen membranes. According to the results, 3% strain on the silicone membranes maintained higher cell viability comparing to control group and 5% strain on the silicone membranes. But, there was no significant difference of migration behaviour between control group and silicone membranes stretched at 3% strain (p=0.27>0.5). The 5% strain caused cell death and also, no significant difference has been found in migration behaviours between control group and silicone membranes stretched at 5% strain (p=0.24>0.5). However, the 3% strain enhanced greater cell migration than the 5% strain (p=0.031<0.5). Based on our preliminary data, different combination of mechanical parameters should be tested in future experiments in order to find the appropriate mechanical loading.

Stress-relaxation tests have shown that silicone and collagen membranes have different mechanical properties. The viscoelastic behaviour of collagen membranes mimics the natural environment of tenocytes better than silicone membranes which show a purely elastic behaviour. The results of tenocyte-seeded collagen membranes have not been shown in this study since the number of experiments was insufficient to present.

5 Conclusion and Future Directions

Mechanical stimulation is currently applied as a method to improve tissue engineered constructs for medical treatment of tendon repair. In this study, we have introduced a perspective to investigate in vitro culture conditions in order to find the effects of mechanical loading on the tenocyte cell migration. To our knowledge, our study is the first one to address the effect of different mechanical loadings on the tenocyte migration. Here, we have examined the effect of cyclic uniaxial stretching on cell migration rate of tenocytes using an in-house tensile bioreactor system which provides real-time information on displacement and forces applied to cells seeded on the carrier materials. Uniaxial mechanical stretching has been applied on the silicone membranes and as well as collagen membranes with different strains and different frequencies. The viability and migration of stimulated cells was compared between unstimulated and stimulated membranes. It has been observed that uniaxially mechanical stretching enhances the cell migration at 3% and 5% strain on silicone membranes. But, an exceeded strain at 5% on the silicone membranes caused the cell death. The results of tenocyte-seeded collagen membranes has not been shown. However, the collagen membrane is considered a promising candidate for tendon tissue engineering due to its biological properties. Therefore, the future aim is to perform more experiments in three dimension with collagen membranes. Also, the mechanical properties of collagen membranes will be better investigated.

Mechanotransduction mechanisms in tenocyte cell migration remain unclear. We therefore aim to track cell migration using different cell migration assays and combine the results with gene and protein expression analysis. Based on initial findings, further experiments need to be performed in order to determine the appropriate mechanical stretching for enhancement of cell migration.

Author Statement

Research funding: The author state no funding involved. Conflict of interest: Authors state no conflict of interest. Informed consent: Informed consent is not applicable. Ethical approval: The conducted research is not related to either human or animals use.

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