Quantification and localisation of damage in rat muscles after controlled loading; a new approach to study the aetiology of pressure sores

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Abstract

To obtain more insight in the aetiology of deep pressure sores, an animal model was developed to relate controlled external loading to local muscle damage. The tibialis anterior muscle (TA) and overlying skin of a rat were compressed between indentor and tibia. Loads of 10, 70 and 250 kPa at skin surface were applied for 2 or 6 h. During half of the 10 and 250 kPa experiments interstitial fluid pressure (IFP) in the TA was measured. The TAs were excised 24 h after load application. Both amount and location of damage were assessed by histological examination using a semi-automated image-processing program. In six of eleven loaded muscles damage was found. The damage was located from superficial to deep muscle tissue in a zone never exceeding the diameter of the indentor. The IFP measurements interfered with the occurrence of damage; application of 10 and 70 kPa loads only caused damage when combined with IFP measurements, whereas IFP measurements increased damage at 250 kPa loads. The results showed that the developed animal model can be used to provoke local damage by applying a controlled load and that the amount and location of damage can be assessed using the newly developed techniques. © 2001 IPEM. Published by Elsevier Science Ltd. All rights reserved.

Keywords: Animal model; Compressive loading; Muscle damage; Pressure ulcers

1. Introduction

Pressure sores are localised areas of tissue degeneration in skin and underlying tissues such as the subcutaneous connective tissue and the muscle tissue. The sores are primarily caused by sustained external (i.e. applied to the skin) mechanical loads. However, preventive measures that aim at reducing these loads, are not always successful in avoiding the occurrence of pressure sores. One of the main reasons is that it is not known how external mechanical loads are transferred to local loads, i.e. local stresses and strains, inside the tissues and how these local loads eventually result in tissue damage. Another difficulty in pressure sore prevention, is that muscle tissue is more susceptible to mechanical loading than skin [1,2]. Pressure sores thus often start to develop in the deep muscle layers near bony prominences and progress towards the surface [3]. Current risk assessment techniques fail to assess the risk of these so-called deep sores at an early stage, as they mainly focus on skin.

The only way to obtain insight in the relation between mechanical loading and initial tissue damage is by applying animal models. Hence, as a guide to pressure sore prevention, many researchers performed animal experiments to determine threshold values for external loads that will result in tissue damage [1,4–7]. In these experiments skin and muscle tissue are compressed between an indentor and bone and the magnitude as well as the duration of the compressive load are varied. Usually the magnitude of the load is defined as the applied load div-
vided by the area of indentation, whereas the onset of pressure sores is defined as the occurrence of tissue damage assessed from histological examination of the tissues. All the studies resulted in inverse relationships between magnitude and duration of the load (Fig. 1), although considerable differences exist between the various relationships. These differences arise from a diversity in laboratory animals, indentation methods, regions of load application, and definitions of initial tissue damage.

A means of overcoming the diversity in animal models and interpreting the differences between the results would be to relate the applied external load to the local loads inside the tissues, whereas these local loads determine the tissue state and hence the occurrence of tissue damage. A comparison of the local loads with the amount and location of tissue damage will then enable a better prediction of the consequences of mechanical loading for the onset of tissue damage. Additional information is therefore needed on the amount and location of tissue damage, while the studies mentioned were limited to determining whether or not damage occurred. Our research approach combines animal experiments, to find the relationship between controlled external loading and local tissue damage, and numerical modelling, providing information about local loads inside the tissue during the experiments.

The aim of the present paper is the development of an animal model that can be used to relate the controlled external loading to local tissue damage and techniques that can be used to quantify and localise the provoked damage. The animal model focuses on muscle tissue, since the most severe deep pressure sores start in muscle. As the reconstruction of the muscle requires a small animal, rats are chosen. Rats have been used as a model for pressure sore formation before [4,8,9].

2. Methods

2.1. Loading procedure

In total, 11 male Brown Norway rats with a weight between 180 and 220 g were used. The rats were anaesthetised using a combination of ketamine (Nimatek, 0.1 ml/100 g) and xylazine (Sedamun, 0.05 ml/100 g) injected subcutaneously. When required, supplemental doses of 0.1 ml ketamine were supplied intraperitoneal. To prevent dehydration, these supplemental doses were mixed with saline solution. Body temperature of the rats was kept between 35 and 37°C using a heating pad and heating lamp. In preparation of the loading experiment, the hairs on the right tibialis anterior (TA) region were cut off, while care was taken not to damage the skin.

The rat was then placed supine in a specially designed loading apparatus, consisting of a unit for fixation of the foot and knee of the right hind limb, and a unit for pressure application (Fig. 2). The foot was fixed on a footplate using adhesive tape and the knee was fixed by clamping the medial and lateral condyles between two concave surfaces. The angles between foot and tibia and between

Fig. 1. Risk curves with regard to pressure sores. A combination of magnitude and duration of load located above a curve resulted in damage, below the curves no damage was detected.

Fig. 2. Loading apparatus consisting of unit for load application and a unit for fixation of the rat’s hind limb.
tibia and femur were approximately 90°. The mechanical load was applied using a pneumatically driven indentor with a rounded contact surface (⌀ 3.0 mm). The edges of the indentor were curved to avoid high stress concentrations. The loading apparatus allowed positioning of the indentor in all directions. During load application, the indentor was placed halfway between foot and knee perpendicular to the skin overlying the TA muscle, at an angle of approximately 40° with the horizontal. In this way, the TA muscle and overlying skin were compressed between the indentor and the tibia (Fig. 3). The location of the indentor relative to the knee, the foot and the tibia, as well as the angle with the horizontal were registered in order to reproduce the loaded area during histological examination.

As the aim of our animal model is to relate controlled external loading to the location and amount of tissue damage, we applied loads that were likely to result in tissue damage, based on values reported in literature. A load of 10 kPa during 2 h was comparable to the lowest reported value resulting in muscle damage, i.e. Kosiak et al. [4] found damage after applying a load of 9.3 kPa during 2 h. The load of 250 kPa was comparable to the highest reported values [7]. The 70 kPa load was comparable to values reported by Daniel et al. [1]. To examine the effect of longer duration and because of the inverse relationship between magnitude and duration of load (Fig. 1), the lowest load was also applied for 6 h. Summarising, loads of 10, 70 and 250 kPa at the skin surface were applied for 2 h (n=3) and a load of 10 kPa was applied for 6 h (n=2).

Interstitial fluid pressure (IFP) measurements can be used to study how external loads are transferred to the tissue [10]. In half of the 10 and 250 kPa experiments (asterisks in Table 1) IFP was measured using a servo-controlled counter pressure measurement system (model 5D, IPM Inc.). For this IFP measurement, a glass micropipette containing 2.0 M NaCl was inserted into the muscle tissue before load application. The micropipette was placed horizontally at a location just beneath the indentor (Fig. 3). A priori, it was assumed that the IFP measurements did not interfere with the occurrence of damage, because the tip diameter of the used micropipettes (2 µm) was small compared to the diameter of muscle fibres (~30 µm). Moreover, previous research demonstrated that IFP measurements with micropipettes did not lead to inflammatory reactions in muscle and skin [11]. Further details on the set-up and results of the IFP measurements are described elsewhere [12].

After the completion of the loading session, the rat was removed from the loading apparatus and observed until it recovered from the anaesthesia. The experimental protocol was approved by the Animal Care Committee of the Maastricht University.

### 2.2. Histology and morphologic analysis

Previous research [4,13] demonstrated that microscopic tissue damage is clearly visible after 24 h. Therefore, 24 h after completion of the loading session, the right TA muscle was excised following perfusion fixation with Bodian’s fluid (90% ethanol, 5% formalin, 5% glacial acetic acid). For one rat the non-loaded contralateral muscle was excised to serve as control sample, whereas for the other rats the non-loaded areas of the muscle served as controls. The muscle was pinned on a piece of cork to avoid shrinkage and the cork was marked to indicate the position of the loaded area. The muscle was dehydrated in a series of alcohol solutions and embedded in plastic (Technovit 7100, Kulzer). To obtain a reference system three parallel holes (0.5 µm) were drilled in the plastic around the muscle. Next, the muscle was cut longitudinally, perpendicular to the direction of load application (section thickness 3 µm). Every 20th section was saved and mounted on an objective slide. As the diameter of muscle fibres is approximately 30 µm, in this way every other muscle fibre was preserved. The samples were stained with toluidine blue to visualise both the cross-striated appearance of muscle fibres and the cell nuclei.

The muscle tissue was evaluated using a Leica Quantimet analysis system coupled onto a Leica DMRA automated microscope. Damage was defined as loss of cross-striation of the muscle fibres and/or the infiltration of inflammatory cells. This damage definition was based on the previous research on the aetiology of pressure sores.
The damaged area in a slice was identified semi-automatically and quantified automatically. The repeatability of the assessment of the damaged area per slice was determined by quantifying the damage in the same slice five times. Every other preserved slice was examined completely, so that the area of damage perpendicular to loading was determined every 120 mm. The slices were recompiled by using the reference system and the volume of the damaged tissue was assessed. The location of the damage was determined by linking the reference system to the location of the loaded area, indicated on the cork.

3. Results

Using the experimental techniques described above, damage was found in six of the eleven loaded muscles. None of the control sites showed any sign of damage. A distinct difference between damaged and undamaged muscle tissue can be observed (Fig. 4). The undamaged tissue has the typical cross-striated appearance of skeletal muscle, while in the damaged tissue the cross-striation has disappeared and mononuclear cells have infiltrated the damaged muscle tissue.

The application of loads of 10 or 70 kPa only caused muscle damage when combined with IFP measurements (Table 2). At a load of 250 kPa damage was present, but considerably more damage developed when the loading was combined with an IFP measurement. Hence, performing the IFP measurement interfered with the occurrence of damage. Considerably more damage resulted from the load of 250 kPa combined with the IFP measurement than from the load of 10 kPa combined with the IFP measurement. During the 6 h loading regime of 10 kPa, muscle damage did not develop.

The identification program that was developed to semi-automatically detect the damaged areas within a slice proved to be effective. The program identified all damaged areas, whereas undamaged areas were not detected (Fig. 5). The assessment of the damaged area per slice was repeatable within 5%.

A three-dimensional reconstruction of the damaged tissue is shown in Fig. 6. Damage was located in a zone around a straight line from the indentor to the tibia. This holds for all loading regimes for which damage was found, including those in which IFP measurements were performed. However, at higher loads the damaged area was broader than at lower loads, but the width never exceeded the diameter of the indentor. Hence, the location of damage proved to be reproducible.

Table 2

<table>
<thead>
<tr>
<th>Applied load (kPa)</th>
<th>IFP measurement?</th>
<th>Volume of damaged muscle tissue (in mm³)</th>
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<tr>
<td></td>
<td>No</td>
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<tr>
<td>10</td>
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<td></td>
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Fig. 4. Longitudinal section of muscle after 24 h after loading showing the typical cross-striated appearance of skeletal muscle (arrowhead), loss of cross-striation of muscle fibres in the damaged area (small arrow) and the infiltration of mononuclear cells (large arrow) (load: 10 kPa + IFP measurement, 2 h).

Fig. 5. Detection of the damaged area within part of a slice (load: 250 kPa, 2 h).
4. Discussion

Former research on the aetiology of pressure sores using animal models, was limited to the question whether or not damage occurred. Furthermore, in these studies, the applied external loads were not related to the local loads within the tissue, whereas these local loads determine the tissue state and thus the occurrence of local tissue damage. To obtain more insight in the effect of mechanical loading on tissue, information is needed on the local mechanical tissue state and the amount and the location of tissue damage.

Therefore, a new rat model has been developed that can be used to relate controlled external loading to local muscle damage. Newly developed techniques enable a semi-automatic quantification and localisation of the provoked damage. Using this approach, valuable insights with regard to the location and amount of damage are obtained. In the present experimental set-up, damage is located in the zone underneath the indentor and ranges from indentor to bone. Although a priori, such a localised volume of damage was not expected, the location of damage proved to be reproducible. In addition, the developed techniques allow an objective assessment of the amount of damage, repeatable within 5%.

The kind of damage found in these experiments, consisting of a loss of cross-striation and infiltration of macrophages, resembles muscle damage described previously [4,8,13]. These damage markers are visible 24 h after the loading of the muscle and are in fact a sign of regeneration of the tissue. Other histological techniques, like immunocytochemical staining or biochemical tests, are being studied to find biological markers capable of signalling early cell damage. In addition, the ability of high-resolution magnetic resonance imaging to assess local muscle damage is currently under investigation. Magnetic resonance imaging will considerably limit the time needed for the quantification and localisation of damage, and thus enable experiments on a larger scale. Furthermore, magnetic resonance imaging is not destructive, as opposed to the histological techniques. Hence, studying development of damage in time as well as clinical applications are possible.

IFP measurements were performed to study the transfer of the applied external loads to the muscle tissue, and so to verify the numerical modelling. Previous research demonstrated that IFP measurements with micropipettes did not lead to inflammatory reactions [11], hence no influence of the IFP measurements was expected a priori. However, this conclusion was not based on histological observations. Our experiments demonstrated that IFP measurements with micropipettes did affect the occurrence of damage. The application of loads of 10 or 70 kPa only damaged the muscle tissue when loading was combined with IFP measurements. At a load of 250 kPa, more damage resulted if the load was combined with the IFP measurement. However, it is remarkable that damage occurred in areas where the micropipette was not inserted, for example near the tibia, while in some areas where the micropipette was present no damage could be found. It is therefore hypothesised that insertion of a non-flexible micropipette in the muscle tissue made the muscle more vulnerable to loading, possibly by introducing extra volume. Kosiak [4] also used needles to measure pressure within the tissues while provoking damage. The lower threshold values he found as compared to other investigators (Fig. 1) may as well be explained from the use of these needles. In future research, separate experiments are to be performed when measuring tissue pressure and provoking damage. In addition, special care has to be taken in clinical studies when needles are introduced in the tissues of patients to measure for example subcutaneous interstitial pressures [14] or muscle oxygen tension [15].
In this research, rats were selected because the reconstruction of the loaded muscle requires a small animal. Rats have been used as a model in the early [4,13] as well as recent studies [8,9]. The soft tissue layers of the rat, in particular the skin, differ from human soft tissue layers [1,7]. This affects the transfer of the mechanical load to the muscle. Therefore, a numerical model will be applied to simulate the experiments and to determine the local stresses and strains within the muscle tissue. Differences in the properties of both skin and subcutaneous fat layer will be accounted for in the numerical model. A comparison of the amount and location of damage, assessed from the experiments, with the local stress and strains, determined by numerical modelling, will then result in local mechanical criteria that are critical for the onset of damage. This approach will supply detailed insight in the loading of the muscle tissue and the occurrence of damage at a cellular level and leads to a better understanding of the aetiology of deep pressure sores.

References