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Original Article

Functional *in vitro* tension measurements of fascial tissue – a novel modified superfusion approach

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Abstract

Introduction: While two laboratory techniques are commonly used to assess the tensile properties of muscle tissue, emerging evidence suggests that the fascial components of these tissues also serve an active role in force generation. Hence, we investigated whether these techniques are sensitive for assessment of fascial micromechanics. **Methods:** Force measurements on dissected fascial tissue were performed either using the classical immersion organ bath or using an improved superfusion approach simulating pulsed pharmacological triggers. Rat deep dorsal fascial strips as well as rat testicular capsule were pharmacologically challenged either with mepyramine or oxytocin. **Results:** The classical immersion technique yielded a lower force response to mepyramine than the superfusion method (median: 367.4 vs. 555.4μN/mm^2). Pause in irrigation before application reduced irregularities during bolus application. The superfusion approach was improved further by the following points: The high sensitivity of the superfusion method to bolus addition was voided by deviation of fluid supply during bolus addition. **Conclusion:** Although both methods demonstrated pharmacologically induced contractile responses in lumbar fascia samples, the modified superfusion method may improve force registrations of slow contracting fascial tissue and minimize artefacts of fluid application.

Keywords: Fascia, Organ Bath, Mechanographic Registration, Superfusion, Immersionmized Mice, Bone Metabolic Marker

Introduction

Fascial tissue forms a body wide, continuous three-dimensional viscoelastic matrix of structural support. Fascia is composed of collagenous connective tissue surrounding and interpenetrating skeletal muscle, joints, organs, nerves and vascular beds[1]. Traditionally, fascial tissue was thought to play an important though passive role in the transmission of force for structural support. Recent data, however, has rebutted a mere passive role of fascia in force transmission. Rather Fascial tissue contains contractile elements that actively generate and modulate force within the tissue and are involved in mechano-sensory fine-tuning[2-5]. Imbalance of this regulatory mechanism causes increased or decreased myofascial tonus, or diminished neuromuscular coordination, which contributes to several musculoskeletal pathologies and pain syndromes[6-9].

Although these data suggest fascial tissue as an important potential contributor to musculoskeletal pathology, *in vitro* data are sparse. This might, at least in part, be explained by the challenging surgical preparation of samples and in particular the need to separate adjunct tissue and to exclude the effects of single muscle fibers. In contrast to phasic force generation registered in skeletal muscle fibres, fascial tissue contains myofibroblasts which show a slow smooth-muscle like contraction pattern[5,10]. The aim of this study was to compare different organ bath methods for *in vitro* investigation of

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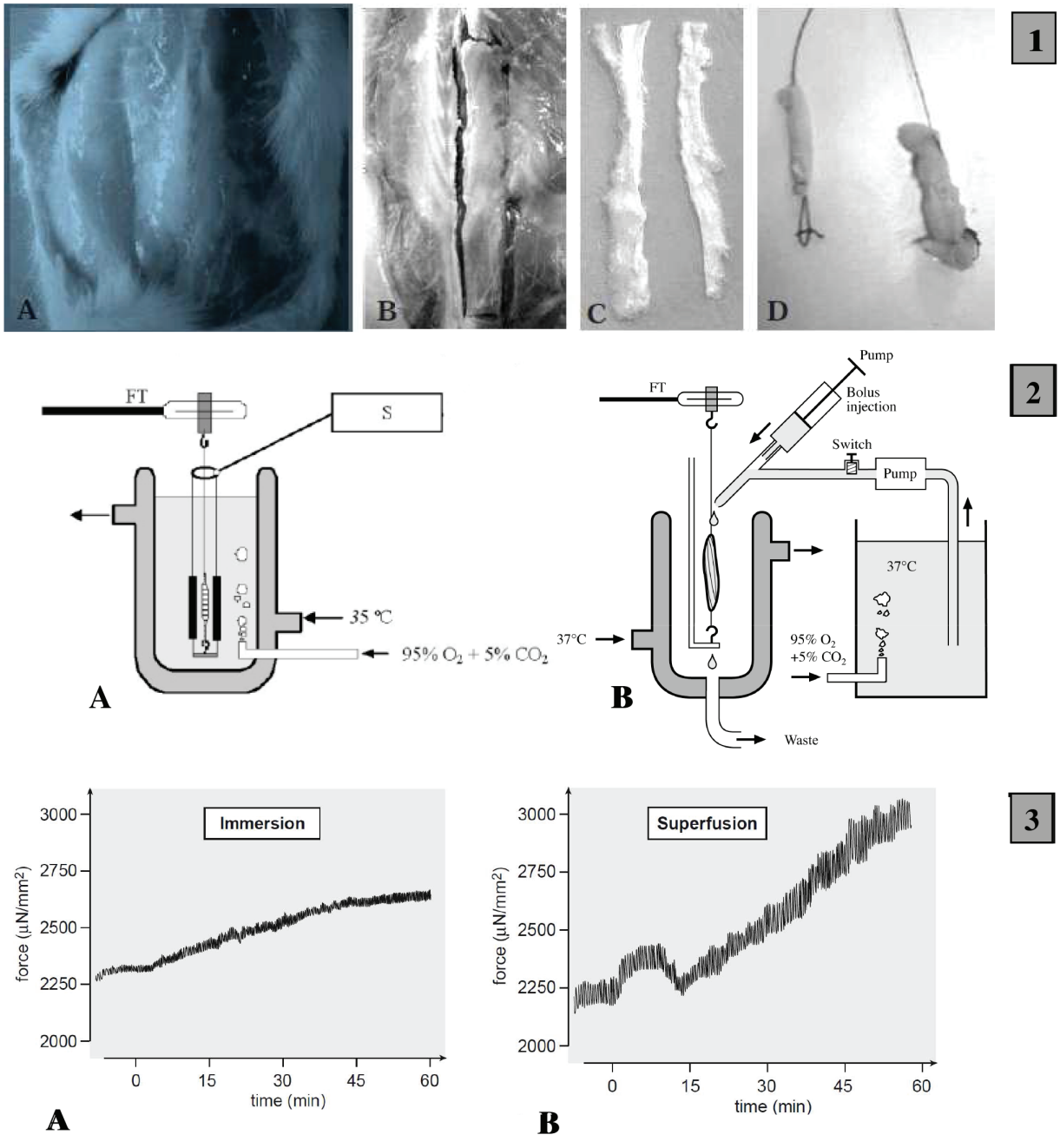

Figure 1.1. Extraction and tissue preparation from rat. **A:** Exposure of deep dorsal fascia. Note the superior-lateral fiber orientation of thoracolumbar fascia. **B/C:** One long piece of fascia is extracted from each lateral side of the thoracolumbar spine. **D:** Each piece is then folded, resulting in double thickness and 50% length reduction, and its new ends were fixated with mercerized cotton.

Figure 1.2. The two compared techniques. **A:** Scheme of immersion organ bath. Through a double walled container the bath is kept at a constant temperature of 35°C. The solution in the bath is aerated with carbogen. The upper end of the tissue is connected with a force transducer (FT). A simulator (S) allows electrical stimulation of the tissue. **B:** Modified superfusion system used in this study. The tissue bundle is irrigated with a continuous downward stream of carbogenated Krebs-Ringer solution. The upper end of the tissue is connected with a force transducer (FT). The temperature of the irrigation solution as well as of the double walled vessel container are adjusted to keep tissue at 35°C. Speed of irrigation solution as well as of bolus addition is regulated with pumps 2 ml/min. During bolus addition (as well as 2 min before and after) the Krebs-Ringer solution irrigation is interrupted for better penetration of the bolus solution into the tissue. Bolus addition occurs with the same speed as the usual irrigation.

Figure 1.3. Concentration response curves of deep fascia in response to 250 µM mepyramine in **A** the immersion system and **B** the superfusion system.

dissected fascial tissue: immersion (physiological conditions: pH, hydration, temperature) versus superfusion (pulsatile effect to imitate blood perfusion).

To compare the different organ bath methods, oxytocin and mepyramine were used as activators of myofibroblasts. Sex hormones have a well documented effect on fascial tissue and are linked to the frequency of injury which might be an explanation for the higher incidence of injury in collagen rich tissue[11]. Furthermore, oxytocin is a potent activator of smooth muscle cell contration[12]. Mepyramine was choosen because of its known effect as activator of myofibroblast contrations[5,13-15]. Mepyramine is a drug with dose-dependent effects of histaminig receptors[16]. While it acts as an inhibitor in millimolar concentrations, it acts agonistic in the micromolar range. This histaminic pathway plays an important role in the course of wound healing and tissue remodelling[17].

Methods

Animals and fascial tissue preparations

Tissue samples of lumbar fascia of 19 mice (type BALB/cJ; 25-35 g) and nine rats (Wistar; 270-450 g) were used according to the local ethics committee of Ulm University. Deep fascial strips (n=20) were taken from a location 5 mm distal and parallel to the spinal cord and before measurement folded once longitudinally (Figure 1.1). For mice the effective length of such a piece was 15-20 mm and for rats 20-38 mm.

Rat testicular capsule (n=9), which is densely populated by myofibroblasts, was used as a positive control to establish the validity of the new experimental system. Briefly, the testis was cut along its greater axis. A 3 mm uncut section at the upper pole was left intact and a piece, 100 mm in length, was removed.

Fresh fascial tissue was kept immersed in Krebs-Ringer solution (Gibco, Karlsruhe, Germany) composed of (mM): 118 NaCl, 3.4 KCl, 0.8 $MgSO_4$, 1.2 KH_2PO_4, 1.1 glucose, 25.0 $NaHCO_3$, 2.5 $CaCl_2$, pH 7.4 and continuously bubbled with carbogen (95% O_2, 5% CO_2, MIT IndustrieGASE, Neu-Ulm, Germany). All visible muscle fibers were removed from fascial tissue with a surgical knife and this was controlled via light microscope with 20x magnification. Contribution of attached skeletal muscle to overall contractile response was further excluded by the effect of ionic challenge with potassium or barium ions. These agents induce contraction of skeletal muscle without affecting the myofibroblasts within tissue[13,18-19]. Finally immunohistochemistry was conducted to confirm that α-smooth muscle actin positive cells, namely myofibroblasts were tested. Paraffin sections were cut into 2.5 µm thick sections and mounted on glass slides coated with 3% saline. These slides were de-paraffinised with xylol and immersed in methanol as decreasing concentrations (100%, 96%, 70%) containing 0.3% hydrogen peroxide for a total of 4 min. After pre-treatment with 0.1% trypsin (Sigma-Aldrich, Steinheim, Germany) for 30 min, sections were blocked with 20% goat serum (Sigma-Aldrich). Finally sections were incubated with mouse monoclonal primary antibody to α-smooth muscle actin (catalogue no. AM128, San Ramon, CA, USA) at 4°C for 1h[5].

Immersion system

At the start, all tissues were arranged in a traditional drainage organ baths (50 ml volume) under the pre-load of 0.5 g. The upper end of the tissue was attached to the free arm of an isometric force-voltage transducer (Model FT03, Grass Instruments, West Warwick, RI, USA) which was connected with a Pentium computer through a bridge amplifier and an analog-digital board (Digidata 1200B, Axon Instruments, Union City, CA, USA). Sampling frequency was 200 Hz.

The fascial tissue was attached to the transducers by a cotton threat at the lower end of the tissue and firmly wrapped with an additional loop attached. The upper end was connected with the hook of the force transducer. The distance between the bath and the force transducer was adjusted until the lower end of the tissue could be looped around the hook at the bottom of the organ bath without causing any pre-stretch of the fascial tissue (Figure 1.2). This arrangement excluded any mechanical compliance in the system which could otherwise have modified the measurements obtained over the many minutes of assessment. The tissues were exposed to a preload and allowed to equilibrate for 30 minutes.

Drugs were added to the calibrated baths in volumes not exceeding 0.2ml. i.e. <1% of the total bath volume. Solvent controls were carried out using ice-cold distilled water. The final concentrations of drugs were established assuming complete mixing of the drug via the disturbance caused by the aeration gas.

Superfusion system

The isolated tissue was suspended in an empty jacketed organ bath with the tension transducer acting as the upper attachment point and the tissue holder as the lower attachment point. The physiological solution was pumped via a peristaltic pump (Watson Marlow Type MHRE 7) at a rate of 2 ml per minute through a warming coil kept at 38°C, which ensured an outflow temperature of 37°C. The physiological solution was aerated before entering the pump. The tissues were surrounded by an air temperature of 37°C and the air was also kept humid by the superfusing physiological solution.

For drug application, a simple device manufactured from micropippete tips was fabricated to form a common snout (Figure 1.2B). The device was positioned 2 cm above the tissue sample with the snout directed downward at 30 degrees to the long axis of the tissue.

Drugs

Oxytocin and mepyramine were all obtained from Sigma Chemicals. All the drugs were dissolved in distilled water, kept on ice and used on the day of preparation.

Statitistical analysis

Differences between the two investigation techniques were compared using the Wilcoxon paired test. P values of less than 0.05 were considered as significant.

Results

Addition of oxytocin to dorsal fascia induced no measurable response with either the immersion (n=2) or superfusion (n=4) methods. In contrast, oxytocin triggered rapid contractions in 6 samples of testicular capsule using the immersion method, but no clear response was detected in the remaining 4 samples of capsular tissues using the same method. Observed contractions with the immersion method peaked within 1-2 min and lasted no longer than 3-6 min.

Addition of mepyramine induced a clear contractile effect on fascial tissue. Examples of mechanographic registrations are displayed in Figure 1.3. A concentration of 250 µM mepyramine resulted in a force increase in rat lumbar fascia of 605 µN/mm^2 ± 371 µN/mm^2 with the superfusion technique and 428 µN/mm^2 ± 286 µN/mm^2 using an immersion bath. The proportion of responsive to non-responsive tests was higher with the superfusion (22 of 32) compared to the immersion bath (13 of 24) technique. Responses of rat testicular capsule were more reliable compared with lumbar samples: 8 of 9 testicular samples contracted in response to 250 µM in a superfusion application, with significantly stronger contraction forces compared to the rat lumbar fascia.

The regular incorporation of inviable control tissue (in which tissue samples had been decellularized by previous exposure to 5 cycles of rapid cooling with liquid nitrogen followed by rapid thawing) in a separate organ bath under otherwise identical treatment conditions enabled the assessment of possible artefacts (e.g. changes in osmolarity, temperature, acidity and minor irregularities in the dripping rate or other perturbations).

The new superfusion arrangement permitted the inflow of the pharmacological agent to be dropped on the tissue though the same exit as the regular solution, while the inflow of the regular solution was temporarily interrupted. This way the administration of the pharmacological test substance could be performed without change in the flow rate, dripping height and drop size of the fluid addition to the tissue. This procedure provided an improved stability and eliminated the large degree of baseline 'noise' which seemed almost unavoidable during the addition of the testing substance in previous superfusion models, due to an increased flow rate during this period or due to sudden changes in the volume or height of the drops.

Discussion

Our functional *in vitro* experiments clearly show contractile responses of excised lumbar fascia bundles to pharmacological triggers. The finding that fascial connective tissue contains contractile elements is suggested to offer new potential treatment options for several pain syndromes. To date, most studies evaluating the force-generating capacity of fascial tissue were conducted in cell culture experiments, which permits valuable investigation of isolated or interconnected myofibroblasts in different environments[20-21]. However, results from cell culture experiments cannot be directly extrapolated to *in vivo* behaviour of the whole tissue. Currently, force measurement experiments of whole tissues are mainly conducted using the immersion bath method. But this method is not very sensitive and does not mimic the natural physiological conditions, such as pulsatile perfusion. Some authors have suggested the superfusion bath is more sensitive in testing contractile responses of fascial tissue, particularly for superficial and deep fascia of rats and guinea pigs[15,22]. Our data would tend to support this concept. The superfusion approach replicates the natural physiological conditions more closely, since the tissue is continuously washed with new solvent elements. This fact ensures a better removal of inhibitory substances which might be produced by the tissue in response to stimulatory agents[23]. Although already introduced by Finkelman in 1930 and improved by several further groups, the superfusion method is usually characterized by a high level of 'measurement noise' due to perturbations arising from the dripping solvent and irregularities during bolus addition[24-27].

Here we included several methodological improvements in the arrangement of the superfusion method, which resulted in lower noise and a better diffusion of the bolus solvent into the tissue. Monitoring of an unviable control tissue permitted exclusion of artefacts, such as the possibility of a bolus application exerting a longer lasting viscoelastic tissue deformation. In conclusion, our data suggests that the modified superfusion approach – if accompanied by our few technical improvements - is superior to the classical immersion bath technique for studying the force-generating capacity of fascial tissue, and allowing examination under more physiological tissue conditions.

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