Journal of Biomechanics ∎ (∎∎∎∎) ∎∎∎–∎∎∎

Contents lists available at ScienceDirect

Journal of Biomechanics

journal homepage: www.elsevier.com/locate/jbiomech
www.JBiomech.com

The association between mechanical and biochemical/histological characteristics in diabetic and non-diabetic plantar soft tissue

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ARTICLE INFO

Article history:
Accepted 18 August 2016

Keywords:
Foot
Diabetes
Subcutaneous
Ulceration
Plantar soft tissue
Adipose

ABSTRACT

Diabetes, and the subsequent complication of lower limb ulcers leading to potential amputation, remains an important health care problem in United States, even with declining amputation rates. It has been well documented that diabetes can alter the mechanical properties (i.e., increased stiffness) of the plantar soft tissue, although this finding is not universal. Similarly, biochemical, and histological changes have been found in the plantar soft tissue, but, as with the mechanical changes, these findings are not consistent across all studies. Our group's work has demonstrated that diabetes increases plantar soft tissue modulus and increases elastic septal thickness. The purpose of the current study was to explore the association between mechanical, biochemical and histological properties. Using previously collected data, a linear mixed effects regression was conducted. The correlations were weak; of the 32 that were tested, only 3 (modulus to septal thickness when location was accounted for, energy loss to total collagen, and energy loss to collagen/elastin ratio) were statistically significant, none with an R^2 greater than 0.10. The main differences in the means were increased tissue stiffness and increased septal wall thickness, both trends were supported in the literature. However, as the correlations were weak, it is likely that another unexamined biochemical factor (perhaps collagen crosslinking) is associated with the mechanical tissue changes.

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1. Introduction

Plantar ulceration and subsequent lower limb amputation are complications of diabetes mellitus that are important clinical problems in the United States. Over 8% of the population has diabetes, disproportionally leading to nearly two thirds of all non-traumatic amputations (nearly 66,000 in 2006) (CDCP, 2011). Recently, there has been a trend of reduced non-traumatic amputation rates, both in veterans (Tseng et al., 2011) and the general population (Belatti and Phisitkul, 2013; Li et al., 2012), likely a result of improved preventative care, increase revascular interventions, and evolving orthopaedic management (Belatti and Phisitkul, 2013). However, in terms of the sheer number of amputations per year (Belatti and Phisitkul, 2013; Tseng et al., 2011), and by the fact diabetic subjects undergo a disproportionate percentage of all amputations (CDCP, 2011; Li et al., 2012), diabetic foot ulceration remains an issue that requires further study.

Ulcer development is a complex and multi-factorial process, with aspects related to autonomic and peripheral neuropathy, poor circulation, and aberrant mechanical tissue loading (Sumpio, 2000). Many groups have studied the mechanical properties of diabetic plantar soft tissue, often with ultrasound devices on living subjects. The findings have not always been repeatable and are sometimes contradictory. Studies have found that diabetic tissue is thicker than normal tissue (Chao et al., 2011; Gooding et al., 1986) and that diabetic plantar skin is harder (Piaggesi et al., 1999). Diabetic plantar soft tissue was shown to have increased energy loss (Hsu et al., 2000, 2002, 2007) but no change in elastic modulus (Hsu et al., 2000, 2002). Conversely, it has been found that diabetic, elderly tissue is stiffer and thinner than non-diabetic, younger tissue, but age may have confounded these findings (Zheng et al., 2000). Others have found that diabetic plantar soft tissue is stiffer at the metatarsal heads, but not at the heel pad (Klaesner et al., 2002). One recent study contradicted earlier work and found no change in thickness in diabetic tissue, but did

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http://dx.doi.org/10.1016/j.jbiomech.2016.08.021
0021-9290/Published by Elsevier Ltd.

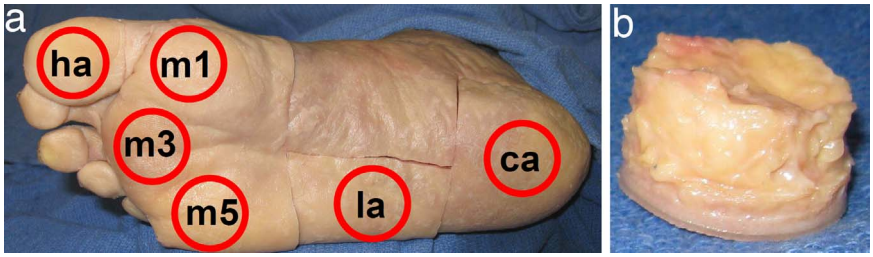

Fig. 1. Specimen locations a) at the hallux (ha), first, third, and fifth metatarsal heads (m1, m3, and m5), lateral midfoot (la), and calcaneus (ca) as well as b) a typical plantar tissue specimen before skin removal. Reprinted from Journal of Biomechanics, Vol. 43, Pai and Ledoux, The compressive mechanical properties of diabetic and non-diabetic plantar soft tissue, 1754 −60, 2010, with permission from Elsevier.

confirm that diabetic tissue was stiffer (Sun et al., 2011). More recently, it has been shown that diabetic tissue has increased Young's and relaxation moduli (Jan et al., 2013). Changing modalities, one group has used indentors and an MRI scanner to determine that older diabetic subjects had increase shear and compressive modulus compared to younger, healthy non-diabetic subjects (Gefen et al., 2001). Magnetic resonance elastography has also been used to demonstrate increased stiffness in diabetic heel pads (Cheung et al., 2006). Our own research group mechanically tested diabetic plantar tissue isolated from cadaveric specimens and demonstrated increased modulus in compression and shear, but no difference in energy loss and very little change in the relaxation properties in either loading mode (Pai and Ledoux, 2010, 2011, 2012).

In addition to the mechanical changes in the diabetic plantar soft tissue, there is also some evidence that the histomorphometric characteristics are altered, although most work has been associated with the heel pad (Buschmann et al., 1995; Jahss et al., 1992; Waldecker and Lehr, 2009). Some of the original studies indicated thicker, frayed septal walls and decreased adipocyte size (Buschmann et al., 1995; Jahss et al., 1992), but a more recent study (Waldecker and Lehr, 2009) found no change in adipocyte size between healthy and diabetic tissue, findings that agreed with our own group's research (Wang et al., 2011). This contradiction might be explained by the fact that the studies from the 1990s used limbs that were amputated due to vascular disease. However, our work has confirmed the finding that thick, damaged elastic septa are found in diabetic plantar soft tissue (Wang et al., 2011, 2016).

Finally, concerning the effect of diabetes on the biochemistry of the plantar soft tissue, it is known that diabetes can induce alterations in the metabolism of the macromolecules present in the body. These biochemical changes are complex and have been found to be dependent on the tissue type and the macromolecule being evaluated (Sternberg et al., 1985), but the evaluation of the biochemistry of diabetic plantar soft tissue is not well understood. Our group has shown that there is no difference in the amount of collagen nor collagen I to III ratios, and minimal differences in the amount of elastin between diabetic and non-diabetic plantar soft tissue (Wang et al., 2016).

In summary, there have been many studies that have explored quantitative differences between diabetic and non-diabetic plantar soft tissue, but the relationship between microstructural characteristics (biochemical and histomorphological properties) and macrostructural characteristics (mechanical properties) is not clear. The purpose of the current study was to explore the direct association between the biochemical/histological characteristics of the plantar soft tissue and the mechanical properties of the plantar soft tissue at six locations beneath the foot. It was our hypothesis that the mechanical characteristics (e.g., increased stiffness or increased energy loss) would be directly associated with biochemical/histological characteristics (e.g., increased elastic septal thickness or increased amounts of collagen).

2. Methods

All cadaveric foot specimens were obtained from the National Disease Research Interchange (NDRI; Philadelphia, PA) and our protocols were all approved by the University of Washington Institutional Review Board.

2.1. Mechanical testing

Two subsets of the mechanical testing data of the plantar soft tissue presented here have been partially published elsewhere with detailed methodologies (Pai and Ledoux, 2010, 2011). Whereas previously we reported on data collected from 4 diabetic and 4 non-diabetic specimens, here we have data from a larger sample including 5 additional non-diabetic donors. Briefly, specimens were collected from six locations, including the: hallux (big toe), first, third, and fifth metatarsal heads, lateral midfoot, and calcaneus (heel) (Fig. 1). Cylindrical specimens (1.905 cm diameter) from these locations were excised and separated from the skin and bone to maintain approximate *in vivo* thickness (3 to 11 mm). Specimens were kept cool on ice until immediately prior to testing.

Testing was conducted in an environmental chamber (near 100% humidity and 35 °C) with each specimen placed between two platens covered in 220 grit sand paper. The apparatus was integrated with an ElectroForce 3200 (Bose Corporation; Minnetonka, MN). A nominal load of 0.1 N was applied and the initial thickness was determined. The target load was based on the donor weight and specimen cross-sectional area, and the required displacement needed to achieve the target load was determined (Pai and Ledoux, 2010). The specimens were tested in compression using triangle waves of varying frequencies (1, 2, 3, 5, and 10 Hz). Each test consisted of 30 triangle waves; after allowing each specimen to precondition, trials 27–29 were sampled at 1000 Hz for the 1, 2, 3, and 5 Hz tests, and 5000 Hz for the 10 Hz tests. Peak stress, peak strain, toe modulus (i.e., slope of the stress-strain curve before the inflection point), modulus (i.e., slope of the stress-strain curve after the inflection point), and energy loss (the area between the loading and unloading curve) were determined for each specimen and frequency. Stress relaxation tests were also conducted (Pai and Ledoux, 2011). Using the same target load and displacement, the specimens were preconditioned with ten 1 Hz sine waves, before undergoing a ramp (0.1 s) and hold (300 s) test. The data were sampled at 1000 Hz, down sampled to 200 Hz, and linear slopes from $t=t_0$ to $t=0.5$, t=10 to t=15, and t=290 to t=300 (all in seconds) were used to approximate the initial, middle, and final relaxation rates.

2.2. Biochemical quantification of collagen and elastin

The biochemical properties of the plantar soft tissue, and the methodologies employed to determine them, have been previously reported elsewhere (Wang et al., 2016) and are described briefly below. Before biochemical quantification of the extracellular proteins, adipose tissue was carefully removed from the skin and defatted.

Collagen content was quantified using an established hydroxyproline assay (Bergman and Loxley, 1963). Defatted samples were lyophilized and subjected to acid hydrolysis with 6 M hydrochloric acid (HCl) for 15 h. The acid was neutralized before collagen quantification. After incubation with Chloramine-T solution and Ehrlich's reagent, absorbance was read at 550 nm (Model 680 microplate reader, Biorad, Hercules, CA). The hydroxyproline content was determined from a standard curve for trans-4-hydroxy-L-proline. Total collagen per sample was calculated using a conversion factor of 6.94, based on the fact that hydroxyproline represents 14.4% of the amino acid composition of collagen in most mammalian tissues (Samuel, 2009).

The Fastin Elastin assay (Biocolor Ltd, Carrickfergus, U.K.) was used to quantify total soluble and insoluble elastin content. Defatted samples were lyophilized, weighed, and subjected to sequential acid digestion with oxalic acid and ethanolic potassium hydroxide. The extracted elastin was quantified as per the manufacturers instructions. Absorbance was read at 515 nm and elastin content was determined from a standard curve for α-elastin.

Please cite this article as: Ledoux, W.R., et al., The association between mechanical and biochemical/histological characteristics in diabetic and non-diabetic plantar soft tissue. Journal of Biomechanics (2016), http://dx.doi.org/10.1016/j.jbiomech.2016.08.021