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Mechanical properties of the lamprey spinal cord: Uniaxial loading and physiological strain

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ABSTRACT

During spinal cord injury, nerves suffer a strain beyond their physiological limits which damages and disrupts their structure. Research has been done to measure the modulus of the spinal cord and surrounding tissue; however the relationship between strain and spinal cord fibers is still unclear. In this work, our objective is to measure the stress–strain response of the spinal cord in vivo and in vitro and model this response as a function of the number of fibers. We used the larvae lamprey (*Petromyzon Marinus*), a model for spinal cord regeneration and animal locomotion. We found that physiologically the spinal cord is pre-stressed to a longitudinal strain of 10% and this strain increases to 15% during swimming. Tensile measurements show that uniaxial, longitudinal loading is independent of the meninges. Stress values for uniaxial strains below 18%, are homogeneous through the length of the body. However, for higher uniaxial strains the Head section shows more resistance to longitudinal loading than the Tail. These data, together with the number of fibers obtained from histological sections were used in a composite-material model to obtain the properties of the spinal cord fibers (2.4 MPa) and matrix (0.017 MPa) to uniaxial longitudinal loading. This model allowed us to approximate the percentage of fibers in the spinal cord, establishing a relationship between uniaxial longitudinal strains and spinal cord composition. We showed that there is a proportional relationship between the number of fibers and the properties of the spinal cord at large uniaxial strains.

1. Introduction

According to the National Spinal Cord Injury Statistical Center (NSCISC), spinal cord injury (SCI) is a serious health problem affecting 250,000 Americans every year, with no effective treatment to regenerate and recover normal function.

An integral element on SCI research is the study of the mechanical properties of the spinal cord (Bueno and Shah, 2008). The importance of studying the mechanical properties can be convened in four concepts: (i) spinal cord fibers have a pre-stress state that could influence the response to an injury, (ii) strain induced on nerve fibers can impact physiological function (Rickett et al., 2011; Shi and Blight, 1996), (iii) mechanical response might depend on the fibers, matrix and meninges (Maikos et al., 2008; Ozawa et al., 2004; Sparrey et al., 2009), (iv) axon regeneration could be enhanced with the appropriate mechanical tension (Smith et al., 2001) and environment (Carlson, 2007; Norman et al., 2009).

The importance of tensile loading on nervous tissue starts during development, where neurons and axons undergo changes in tension during growth and network formation (Van Essen, 1997; Anava et al., 2009). Therefore, it is no surprise that fiber strain has such an important role in the study of spinal cord function, injury and repair (Sato and Swenson, 1984; Gruner, 1992; Meade et al., 2004; Teng et al., 2002; Bueno and Shah, 2008). Unfortunately human studies are limited to computer simulations (Czyz et al., 2008; Li and Dai, 2009; Greaves et al., 2008) and experimental techniques are limited to cadavers, with great variation of the modulus (0.5–1.5 MPa) (Bilston and Thibault, 1996; Sparrey et al., 2009; Oakland et al., 2006). Other animal models have been used, such as bovine (0.94–1.66 MPa) (Ichihara et al., 2001), cats (0.255 MPa) (Chang et al., 1988), dogs (0.04–0.215 MPa) (Chang et al., 1981; Hung and Chang, 1981). A significant amount of force experiments have been done in rodent models, which has allowed us to better understand the biomechanics of the spinal cord (1.2 MPa) and the physiological and in vitro effects of applied forces on nerves (e.g. stretching) (Maikos et al., 2008; Russell et al., 2012; Abe et al., 2002; Bora et al., 1980; Ichimura et al., 2005; Jou

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et al., 2000; Spiegel et al., 1993; Pfister et al., 2004; Pfister et al., 2006). Our animal model of choice, lamprey (*Petromyzon Marinus*), is a basal vertebrate model used to study spinal cord regeneration (Cohen et al., 1989; Cohen et al. 1988, 1986; Buchanan and Cohen, 1982; Lurie and Selzer, 1991a, 1991b; Yin and Selzer, 1984, 1983; Oliphint et al., 2010) and animal locomotion (Cohen et al., 1990; Cohen, 1988, 1987; Cohen et al., 1982; Cohen and Wallen, 1980; Tytell et al., 2010). We used the lamprey for its regenerative capabilities after SCI and thus their usefulness in understanding this process. We decided to investigate the mechanical properties in vivo and in vitro of uninjured spinal cords; using a controlled environment with freshly obtained spinal cords.

The objective of this work was to measure the response of the spinal cord to uniaxial tensile longitudinal loading and to create a model that describes this behavior as a function of the fraction of fibers. We obtained stress–strain curves and in vivo uniaxial tensile longitudinal strain (from hereon also referred as uniaxial strain) measurements that allowed us to acquire the soft tissue properties of the spinal cord. We adapted a mathematical model based on composite materials, to explore the dependence of uniaxial strain with respect to the fiber and matrix composition. This study provides a baseline for the future analysis of uninjured, injured and regenerated lamprey spinal cords in order to comprehend the relationship between biomechanics and spinal cord regeneration.

2. Methods

2.1. Surgery and isolation of spinal cord

Spinal surgery was made after first anaesthetizing the animal with MS222 (100 mg/l, tricaine methylsulfonate, Argent Labs, Nebraska, USA). For isolation of the spinal cord, the animal was rapidly decapitated, pinned and the spinal cord exposed by removal of the dorsal musculature. The spinal cord was removed and maintained in HBSS solution (Hank's Balanced Salt Solution, Invitrogen, California, USA). Then uniaxial loading was applied 5 min after isolation to obtain the tissue stiffness. Spinal cord sections were selected as follows: Head (3 cm down at the level of gills), Middle (3 cm at mid-section) and Tail (3 cm after the dorsal fin appears).

In the lamprey, the meninges which wrap and protect the nervous tissue (Nieuwenhuys et al., 2008), can be removed without sacrificing spinal cord tissue, avoiding inconsistency in our results. Removal was done with a pair of tweezers (0.025 mm × 0.015 mm tips, Dumont #5, Electron Microscopy Sciences, Philadelphia, USA), by gradually detaching the meninges with an upward motion while holding the spinal cord static. Animal maintenance and surgical procedures were approved by the University of Maryland's Institutional Animal Care and Use Committee, IACUC.

2.2. In vitro tensile loading experiments

Uniaxial loading experiments were carried out using a custom-made set up (Fig. 1a) that consists of a set of micromanipulators (Model M-460A-xyz and SM-13, Newport, Montana, USA) that controlled the overall tissue displacement and a force transducer (Force range 0 to 10.0 mN, Sensitivity 1.0 mN, Resolution 200.0 nN, Linearity ±0.2% of full scale over 50% of full scale, ±1.0% of full scale over full scale, Maximum Overload Force 100.0 mN, Model 405A, Aurora Scientific, Ontario, Canada). Freshly isolated spinal cords (3–5 cm long) were placed in a chamber filled with lamprey saline solution (115 mM NaCl, 2.0 mM KCl, 2.6 mM $CaCl_2$, 1.8 mM $MgCl_2 \cdot 6H_2O$, 2.0 mM HEPES, 4.0 mM Glucose, Sigma-Aldrich, Missouri, USA), to avoid dehydration, and gripped using surgical grade fibers to both wires connected to the micromanipulator and force transducer respectively (Fig. 1b). The gripping method was modified from (Shah and Lieber, 2003), a double overhand suture loop was used to secure the tissue without slippage; while fibers were compressed at the knot, the absence of breaks or slippage allowed consistent behavior from trial to trial. Readings were taken in the steady state;~10 min after the spinal cord was stretched approximately 0.5 to 2 mm at a time using the manipulator. Each force value (F) was then divided by the area of the spinal cord (A) to obtain the stress ($\sigma = F/A$). For the stress–strain calculations, the spinal cord was assumed a cylinder. While the cross section of an in vivo lamprey cord is elliptical, this is not the case for the isolated spinal cord. After isolation, we placed the spinal cord in the micromanipulator and rotated it at the microscope. We observed one constant width throughout the rotation, which indicated that once isolated the spinal cord is circular, therefore we assumed the spinal cord had a cylindrical shape. The strain was obtained by dividing the change in spinal cord length ($L-L_0$) over the unloaded length (L_0), ($\varepsilon = L-L_0/L_0$). The results of 10 spinal cords without meninges, 5 samples for each section (Head, Middle and Tail) and 5 cords with meninges were plotted as a stress vs. strain curve. Statistical analysis was performed using Student's t-test and factorial analysis of variance (ANOVA) with a $p < 0.05$.

2.3. In vivo measurement of spinal cord strains

In vivo experiments were made by first anaesthetizing the animal with MS222 (100 mg/l). The spinal cord was exposed and the musculature pinned open, but not removed. Two polyester glint marks were placed at the start and end of the cord and the distance between them measured, and recorded as the physiological length. Then, both points were cut with a scalpel and the shortened cord was measured and recorded as the in situ unloaded length, this experiment was repeated for 5 different animals and strain between the physiological length and the in situ unloaded length was calculated, we will call this the physiological uniaxial strain.

Similarly, in order to understand the physiological threshold levels, we repeated this experiment by placing the animal into the most common swimming position. The swimming position was defined when the body had two areas of maximum curvature, each localized at approximately 25% and 75% of its own length (Fig. 2a). The animal was anesthetized, the musculature pinned open and marks are placed in the spinal cord. We placed two marks separated approximately 1 cm in the Head section (below the gills), two marks at the Middle section (3 cm below the Head section) and two marks at the Tail section (3 cm after the dorsal fin). First the animal was placed straight (at rest, Fig. 2b) and the length between each marker was measured. Then the animal (while anesthetized) is moved into the swimming position and the longitudinal length between each marker was measured and the local uniaxial strain is calculated. Even when anesthetized, the animal will adopt the swimming position almost automatically, thus the amount of force needed to bend the animal is minimal. Statistical analysis was performed using Student's t-test and factorial analysis of variance (ANOVA) with a $p < 0.05$.

2.4. Histology and fluorescence staining of spinal cord

To better understand the uniaxial strain response of different sections in the spinal cord, we used histology to measure the number of giant fibers at the Head, Middle and Tail sections. Spinal cords were fixated using Mirky's Fixative solution (National Diagnostics, Georgia, USA), following dehydration in ethanol series; Head, Middle and Tail sections were embedded in paraffin and a series of 5 (20 μm thick) slices obtained and stained with toluidine blue (Sigma Aldrich, Missouri, USA). Microscopy images were obtained for each series of slices and the Müller and Mauthner's giant fibers (Rovainen, 1976) were identified and the number measured using ImageJ (U.S. National Institutes of Health, Maryland, USA).

Fresh spinal cords, with and without meninges were stained using fluorescent wheat germ agglutinin (WGA, Molecular Probes, Oregon, USA). After isolation from the body and removal or not of the meninges, spinal cords were incubated for 5 min at 4 °C in 10 μg/ml concentration of WGA. The spinal cord was washed twice in HBSS solution and incubated for 5 min at 37 °C in HBSS.

3. Results and discussion

3.1. Mechanical properties of the spinal cord (in vivo and in vitro)

Using our custom tensile loading apparatus, we measured the strain–stress response of isolated spinal cords and different sections (Head, Middle and Tail). We found that the lamprey spinal cord behaves like a soft tissue material (Humphrey, 2002) (Fig. 2d) with a stress–strain curve characterized by two regions. The low strain region,~0–18% strain has a modulus of ~0.015 MPa (Table 1). As the strain increases, the curve changes slope, reaching a higher strain region with a 0.5 MPa modulus (Tables 1 and 2). The intersection between the low and high modulus, called the critical strain, was found to be an average 18% strain (Table 1).

After the cord was isolated from the body, we observed a contraction in the unloaded length, which indicated the existence of a pre-stressed state. Using markers on the spinal cord, we found that the in situ cord retracts an average of 10% of its physiological length. We considered this value as the physiological uniaxial strain at rest. Next, we analyzed the local uniaxial strains at the Head, Middle and Tail during swimming. We found local