In situ microextraction method to determine the viscosity of biofluid in threadlike structures on the surfaces of mammalian organs

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We report a method to measure the viscosity of μL volumes of biofluid obtained from threadlike structures (NTSs) on the surfaces of mammalian (rabbit) internal organs. The fluid was mechanically microextracted *in situ* from NTSs on the organ surfaces by a glass capillary connected to an extractor. From the Brownian motion of the 0.8 ± 0.1 μm diameter granules in the extracted fluid, the fluid viscosity was determined to be 1.4 ± 0.1 mPa·s at room temperature. This viscosity is comparable to the viscosity of rabbit blood plasma.

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The surfaces of internal organs of mammals can harbor novel threadlike structures (NTSs) [1,2]. NTSs have been considered as parts of a circulatory system that is thought to be the anatomical basis of classical acupuncture meridians since its first observation in 1963 by Bonghan Kim [3]. NTSs, also termed Bonghan ducts (BHDs), consist of three subsystems. The first is comprised of superficial BHDs located in the skin [4], the second consists of intravascular BHDs located inside large blood and lymphatic vessels [5–8], and the third comprises BHDs on the surface of internal organs [9,10]. NTS can contain swollen regions called Bonghan corpuscles (BHCs). Superficial, organ-surface, and intravascular BHCs also have been reported [4,9,11].

The interior of a NTS contains a specific liquid [Bonghan liquid (BHL)]. BHL is composed of hormones (adrenalin and noradrenalin) [12,13], hyaluronan, free amino acids, and 0.5–2 μm diameter DNA-containing granules [9,14,15]. Liquid can flow through BHDs and BHCs at a rate of 0.3 mm/s [16].

Concerning microcirculation, the viscosity of blood plasma is a major influence on endothelial shear stress, and is used as a marker for cardiovascular diseases [17]. Since BHL has a crucial role in microcirculation related to the therapeutic effects of acupuncture, precise measurement of BHL viscosity is important [3,18,19]. However, viscometry, rheometry, or other measurements are hampered by the extreme difficulty in obtaining a sufficient volume of BHL [12].

In this article, we report a method to measure the viscosity of μL volumes of biofluid (BHL) mechanically microextracted *in situ* from NTSs on rabbit organ surfaces by a glass capillary connected to an extractor. Viscosity determined from the Brownian motion of granules in the extracted BHL at room temperature was comparable to that of rabbit blood plasma.

New Zealand White rabbits of about 1.8 kg were housed in a temperature-controlled environment (23 °C) with 60% relative humidity and a 12-h light/dark cycle. The animals had ad-libitum access to food and water. The procedures involving the animals and their care conformed to institutional guidelines, which were in full compliance with current international laws and polices (Guide for the Care and Use of Laboratory Animals, National Academy Press, 1996). The appropriate guidelines for experiments with animals were followed. The rabbits were anesthetized with urethane (1.5 g/kg) administered intraperitoneally, and all surgical procedures were performed under general anesthesia. About 1 h after anesthesia, the abdominal wall of each rabbit was dissected under deep anesthesia. The large vessels in the skin of the abdomen and the thorax were held by hemostats for hemostasis so that blood flow over the organ surfaces was minimized. Bonghan corpuscles (BHCs) are thickened portions of BHDs that are relatively easily located. We searched for BHCs on organ surfaces using small surgical instruments such as iris scissors, microforceps, and needles for manipulation of samples positioned in a Model SZX12 stereoscopic microscope (Olympus, Tokyo, Japan). For the mechanical extraction of BHL, a glass capillary (tip diameter of 30–50 μm) connected to an IM-9C pneumatic injector-extractor (Narishige Scientific Instrument Lab, Tokyo, Japan) by a polyethylene tube and a capillary holding unit (IM-H2 Injection Holder; Narishige Scientific Instrument Lab) was injected into a BHC *in situ* by a *x*-*y*-*z* micromanipulator. The extractor supplied negative pressure to the internal part of the corpuscle through the capillary for 10 min, during which time saline was sprayed onto the organs to prevent drying of the tissue. After extraction of the liquid, the glass capillary was immediately transferred to the stage of a Model BX71 phase contrast microscope (Olympus) for observation at room temperature (approximately 25 °C). Images were recorded using a Model DP70 CCD camera (Olympus) attached to the microscope (10 frames/s, frame rate of 1360 $\times$1024). The images were analyzed using a MATLAB-based in-house image processing program. The program first recognized the boundaries of the objects in the images, then tracked the movements of the center of mass of each object over time, and calculated statistical quantities such as size, velocity distributions, and mean-square displacement of the moving objects.

BHDs on the surface of mammalian internal organs were thin (20–100 μm), semitransparent, and freely movable strands that were irregularly fixed on the peritonea. BHCs with elongated-ovoid shapes averaging 200 μm–2 mm in

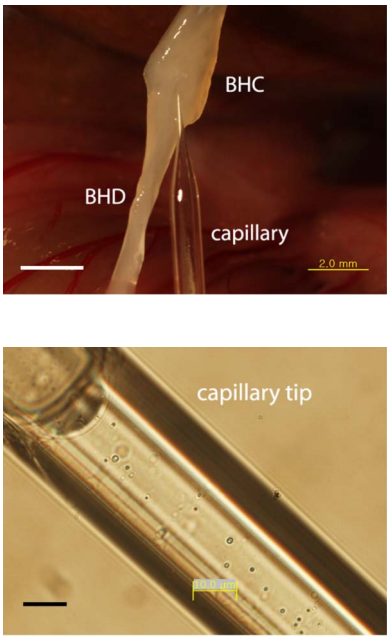

FIG. 1. (Color online) Images of fluid extraction and the presence of granules. (a) Stereomicroscopic image of a NTS. A glass capillary was carefully injected *in situ* into the BHC by means of an *x*-*y*-*z* micromanipulator under stereomicroscope guidance, and negative pressure was loaded in the capillary to extract the intrinsic liquid in the NTS. Scale bar represents 2 mm. (b) Phase contrast microscopic image of the tip of a glass capillary after liquid extraction from the NTS shown in (a). It was possible to observe the granules inside the glass capillary without distortion or defocusing in the 20–50 μm diameter tip region of the capillary diameter. Several granules with a diameter of 0.8 ± 0.1 μm could be observed. They were moving randomly in the glass capillary and could be imaged in-focus only in the tip-part of the capillary (inner diameter approximately 20 μm), in which the effect of the optical ray deflection by the capillary wall was minimized. Scale bar represents 10 μm.

diameter were evident along the BHDs, and the corpuscles were linked at both ends to BHDs. NTSs containing both BHDs and BHCs were observed on the surfaces of different internal organs including the liver, stomach, small and large intestines, and bladder.

We successfully extracted liquid from BHCs with extraordinarily careful manipulations of the glass capillary and extractor [Fig. 1(a)]. It was possible to observe the granules inside the glass capillary without distortion or defocusing in the 20–50 μm diameter tip region of the capillary diameter. In the extracted liquid, 3–30 granules could be observed under phase contrast microscopy [Fig. 1(b)]. The granules displayed a uniform diameter of 0.8 ± 0.1 μm, which is consistent with previous reports [9,14,15]. The granules moved randomly in the glass capillary and could be imaged in-focus only in the tip-part of the capillary where the inner diameter was about 20 μm; in this region the effect of the optical ray deflection by the capillary wall was minimized. In regions of the capillary where the diameter was greater, distortion of the light prevented focusing. Because of the curvature of the glass capillary, the edge area of the capillary was also not focusable.

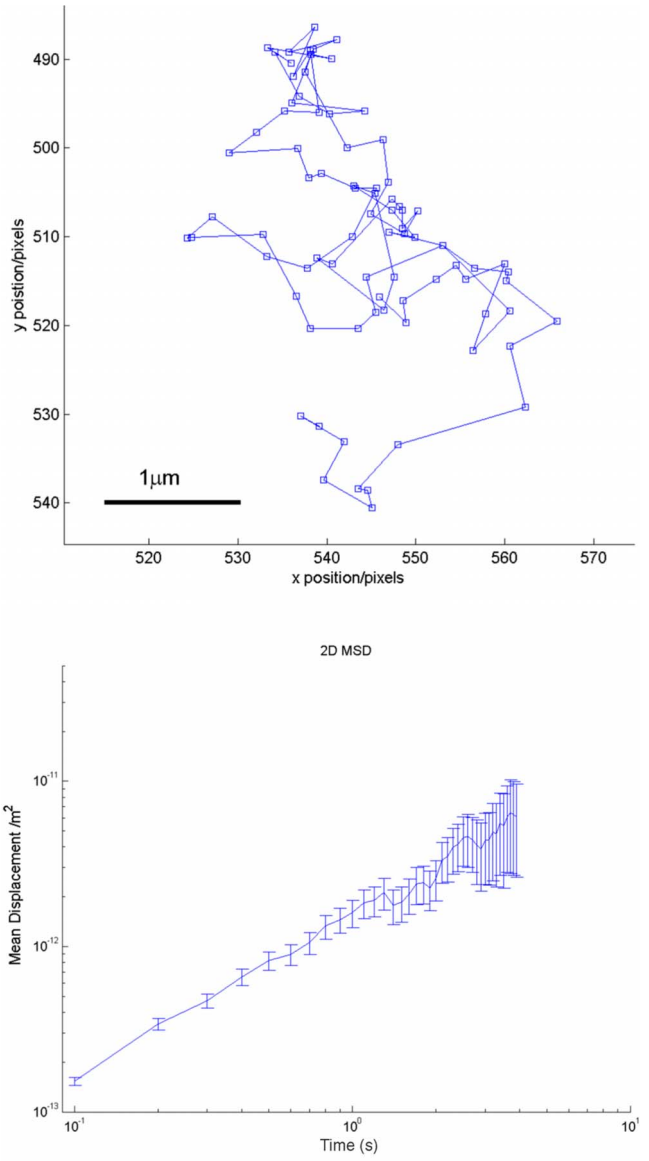

FIG. 2. (Color online) Two-dimensional (2D) plots of granule motion. (a) An example of the 2D trajectory of a granule in the liquid extracted from NTS showing a typical random motion in plane at room temperature (25 °C). The *x* and *y* axes represent the *x* and *y* positions in pixels, respectively. Scale bar=1 μm. (b) Plot of the 2D mean-squared displacement of the granules with time. The *x* and *y* axes represent time (s) and mean squared displacement (m^2), respectively. The plot shows a well-fitted linear curve having a slope of $1.5 \pm 0.1 \times 10^{-12}$ m^2/s, consistent with Brownian motion of the granules.

Figure 2(a) shows an example of the trajectory of the random motion of the granules. The mean square displacement (MSD) is defined as $\langle \Delta r^2(t) \rangle = \langle |\vec{r}(t_0+t) - \vec{r}(t_0)|^2 \rangle_{t_0}$, where $\vec{r}(t_0)$ is the position of the granule at time t_0. The plot of the two-dimensional mean-square displacement of the granules with time showed a well-fitted linear curve with a slope of $1.5 \pm 0.1 \times 10^{-12}$ m^2/s [Fig. 2(b)] indicating that the motion of the granules at room temperature (25 °C) was exactly Brownian and purely diffusive. The MSD is related to the diffusion constant D by $\langle \Delta r^2(t) \rangle = 4Dt$ in two dimen-

sions, producing a diffusion constant $D=3.8\pm0.2\times10^{-13}$ m^2/s. To calculate the viscosity of the fluid, we used the Stokes-Einstein equation $\eta=\frac{2k_BT}{6\pi dD}$, where η, d, T, and k_B represent viscosity, particle diameter, temperature, and Boltzmann constant, respectively. In our experiment, $d=0.8\pm0.1$ μm and $T=298$ K, producing $\eta=1.4\pm0.1$ mPa s.

The present observation of the Brownian motion of the BHL granules is consistent with the finding that at room temperature the velocity distributions of living microparticles does not deviate from the Maxwell-Boltzmann distribution [20]. If temperature could be controlled in vital conditions (38 –39 °C), the presently described capillary extraction method could be used to observe non-Brownian motion of the granules [21].

It is notable that the viscosity of the liquid extracted from rabbit NTSs (approximately 1.4 mPa s) is comparable to that of blood plasma (approximately 1.3 mPa s) [17]. This result with the viscosities of whole blood under three share rates is shown in Fig. 3. The viscosity of the NTS liquid was significantly lower than that of whole blood and was similar with that of blood plasma. Since it is known that the NTSs can transport liquid [16], the comparable viscosities of NTS liquid and plasma is an important evidence implicating NTSs in microcirculation. It is conceivable that NTS viscosity might be germane in certain circulatory diseases.

Our method for determining biofluid viscosity can be also applied to the measurement of viscosity using an extremely small volume (μL) of body fluids *in vitro*, *in vivo*, or *in situ*. However, the optical and electrostatic effects of the glass capillary wall on light deflection and the motion of granules should be further investigated to allow a more precise determination of viscosity. Furthermore, influence on the motion change of the NTS granules of physical parameters such as temperature, light irradiation, and electromagnetic fields should be examined to clarify the biophysical properties of the granules in NTSs.

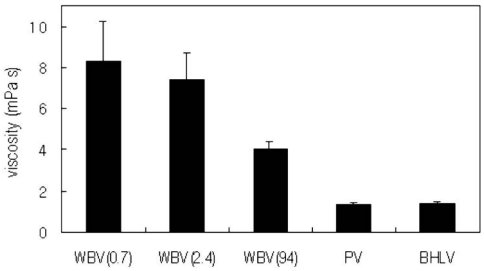

FIG. 3. Biofluid viscosities of rabbit. The data was reconstituted from a previous data table (Windberger *et al.*), with the value of the NTS liquid (BHLV). The viscosity of the liquid extracted from NTSs (approximately 1.4 mPa s) is comparable to that of blood plasma (approximately 1.3 mPa s). The viscosity of the NTS liquid (BHLV) is significantly lower than that of whole blood (WBV) and is similar with that of blood plasma (PV).

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