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The best approach: Homogenization or manual permeabilization of human skeletal muscle fibers for respirometry?

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ABSTRACT

The number of studies on mitochondrial function is growing as a result of the recognition of the pivotal role of an intact mitochondrial function in numerous diseases. Measurements of oxygen consumption by the mitochondria in human skeletal muscle are used in many studies. There are several advantages of studying mitochondrial respiration in permeabilized fibers (Pfi), but the method requires a manual procedure of mechanical separation of the fiber bundles in the biopsy and chemical permeabilization of the cell membrane. This is time-consuming and subject to interpersonal variability. An alternative is to use a semiautomatic tool for preparation of a homogenate of the muscle biopsy. We investigated whether the PBI shredder is useful in preparing a muscle homogenate for measurements of mitochondrial respiratory capacity. The homogenate is compared with the Pfi preparation. Maximal respiratory capacity was significantly reduced in the homogenate compared with the Pfi from human skeletal muscle. A marked cytochrome *c* response was observed in the homogenate, which was not the case with the Pfi, indicating that the outer mitochondrial membrane was not intact. The mitochondria in the homogenate were more uncoupled compared with the Pfi. Manual permeabilization is an advantageous technique for preparing human skeletal muscle biopsies for respirometry.

Mitochondria play a central role in different physiological and pathological conditions [1–6], as well as in the aging process [7,8], and have been studied intensively during the past decade. Critical information on mitochondrial integrity and function can be obtained when coupled mitochondrial respiration and ATP production are assessed. Different approaches can be used to evaluate mitochondrial function ex vivo [9,10]. In 1956, Chance and Williams developed a technique where mitochondria were isolated from skeletal muscle [11]. Some decades later, Saks and coworkers developed the permeabilized fibers (Pfi)[1] technique [12,13]. It has been reported that the vast majority of mitochondria (~95%) are present after permeabilization and still situated within the cell [12,14], whereas only approximately 20 to 40% of the mitochondria are present in a preparation of isolated mitochondria (Imt) [8,15]. As deduced from a variety of studies on skeletal muscle (e.g., human, rat) and rat heart, Pfi and Imt yield comparable estimates of respiratory capacity (within experimental error) when normalized to tissue mass or mitochondrial protein [16,17]. Recently, it was reported that critical differences exist between the two techniques not only in regard to mitochondrial respiratory capacity [18–20]. A difference in long chain fatty acid oxidation between Imt and Pfi has also been reported [21]. Both techniques require a skilled person to isolate the mitochondria or to prepare the fibers. Due to interpersonal variation, it is recommended that the same person perform the procedure in a series of experiments. Both techniques are quite time-consuming (1–2 h to prepare the sample [16,19]). Recently, a new technique was developed to prepare tissue homogenate using a shredder, which is a mechanical grinder that homogenizes the tissue [22]. This technique is more time-efficient, and the preparation is semiautomatic, thereby minimizing the personal and technical variation, which makes the procedure more standardized and probably more reproducible. In a study by Gross and coworkers, kidney and skeletal muscle from rats were studied, comparing a homogenizer (Imt) with a shredder, and the authors reported similar respiratory acceptor control ratios (RCRs) except when the highest pressure cycle was added on top of the shredder technique [22]. The current study was undertaken to investigate whether a homogenate from a human skeletal muscle biopsy generated by using the PBI shredder is comparable to permeabilized muscle fiber preparation from the same biopsy with respect to the respirometry measurements. Furthermore, skeletal muscle tissue from mice was also tested to see whether species differences might exist.

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[1] *Abbreviations used:* Pfi, permeabilized fibers; Imt, isolated mitochondria; RCR, respiratory acceptor control ratio; BMI, body mass index; W_w, wet weight; H_2O_2, hydrogen peroxide; CI, complex I; ADP, adenosine diphosphate; OXPHOS, oxidative phosphorylation; FCCP, *p*-trifluoromethoxyphenylhydrazone; CV, coefficient of variation; EM, electron microscopy.

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Materials and methods

Subjects

The subjects were 9 overweight (body mass index [BMI] = 28 ± 1 kg/m^2) individuals (3 females and 6 males) aged 58 ± 4 years. Subject characteristics are shown in Table 1. The ethics committee of the municipality of Copenhagen and Frederiksberg, Denmark, approved the study protocol. Oral and written consent was obtained from each participant in accordance with the Helsinki Declaration.

Experimental procedure

Subjects arrived to the laboratory in the morning (8–9 am). Subjects were placed in a bed, and a muscle biopsy was obtained from musculus vastus lateralis under local anesthesia (Lidocain 5%, ~2–3 ml) using a Bergström needle modified for suction [23]. The muscle biopsy was placed in a relaxing buffer as described previously [1] and was analyzed immediately for mitochondrial respiratory capacity using a high-resolution respirometer (Oroboros Instruments, Innsbruck, Austria) with the respiratory protocol described below.

Preparation of permeabilized fibers and homogenate (shredder)

Permeabilized fibers

Preparation of the Pfi was done as described previously [1]. Briefly, the muscle fibers were gently dissected on ice using sharp needles and were permeabilized in a relaxing buffer with saponin (50 µg/ml) for 30 min. BIOPS contains CaK_2EGTA (2.77 mM), K_2-EGTA (7.23 mM), Na_2ATP (5.77 mM), $MgCl_2{\cdot}6H_2O$ (6.56 mM), taurine (20 mM), Na_2Phosphocreatine (15 mM), imidazole (20 mM), dithiothreitol (0.5 mM), and Mes (50 mM) at pH 7.1 and 0 °C. This was followed by rinsing the Pfi twice for 10 min in a mitochondrial respiration medium (MiR05 [19]), also on ice. MiR05 contains EGTA (0.5 mM), $MgCl_2{\cdot}6H_2O$ (3 mM), K-lactobionat (60 mM), taurine (20 mM), KH_2PO_4 (10 mM), Hepes (20 mM), sucrose (110 mM), and bovine serum albumin (1 g/L) at pH 7.1 and 37 °C.

Shredder homogenate

The PBI shredder SG3 (Pressure BioSciences, South Easton, MA, USA) was used in the study. This is a mechanical homogenization system that uses a twisting motion to disrupt the tissue. It is capable of producing three different reproducible forces on the homogenized tissue. This system should be able to eliminate operator-specific differences (interpersonal variation).

The skeletal muscle fibers were weighed (wet weight, W_w) on a calibrated scale (Mettler Toledo, XS105) before being transferred into the narrow ram side of the prechilled (kept on ice) shredder tube (Fig. 1) and distributed evenly on the lysis disk at the narrow ram side of the shredder tube. The shredder ram was inserted with a twisting motion to press the sample between the serrated surface and the lysis disk (Fig. 1). Then 500 µl of mitochondrial medium buffer (MiR06: MiR05 + catalase, 280 U/ml) was added at the wide side of the shredder tube and the shredder ram (Fig. 1) was inserted. The total volume of buffer should not exceed 0.8 ml. The prechilled shredder tube was then placed in the prechilled shredder base. When using the shredder, it is possible to apply three different forces on the tissue: weak (position 1), medium (position 2), and strong (position 3). The homogenate was prepared using different protocols described later in this section. After the shredding procedure, the homogenate was removed by unscrewing the shredder cap and was transferred into a 15-ml Falcon tube on ice using a pipette. A minor amount of muscle tissue was not forced through the lysis disk with the shredding procedure. This muscle tissue was removed, weighed, and subtracted from the initial weight of the muscle fibers so that the precise amount of shredded tissue is known and subsequently measured in the Oxygraph-2k (Oroboros Instruments) (Table 3). The shredder tube, including the lysis disk, was then rinsed carefully with cold MiR06 to recover any residual sample to a total volume of 5 ml of homogenate. A specific shredder tube ram tool (Oroboros Instruments) was used to remove the shredder ram (Fig. 1) for rinsing the lysis disk from both sides [24].

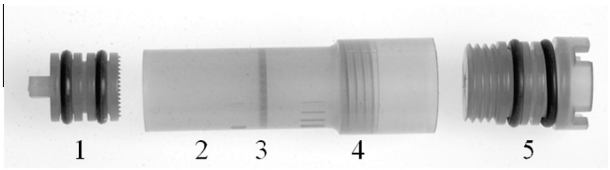

Fig.1. FT 500-PS pulse tube. (1) The rotating ram that grinds the tissue with the serrated surface is shown. (2) The sample is placed between the ram (1) and the lysis disk (3). (3) The tissue is forced through the lysis disk during the homogenization procedure. (4) The homogenate is then obtained from the upper chamber that contains the buffer (MiR06). (5) The cap is shown.

The shredder protocols were as follows:

Protocol 1 (SH1): 2 × 10 s on step 1 (weak)
Protocol 2 (SH2): 2 × 10 s on step 2 (medium)
Protocol 3 (SH3): 2 × 10 s on step 3 (strong)
Protocol 4 (SH4): 1 × 10 s on step 1 (weak) + 1 × 5 s on step 2 (medium).

A 10-s pause was held in between each step in all of the different protocols. When 10-s intervals were applied, the direction of the shredder was changed after 5 s.

Measurements of respiratory oxygen fluxes

Each protocol was made in duplicate after hyperoxygenation (400 µmol/L). The Pfi were weighed (between 1.0 and 2.5 mg for each respirometer chamber [W_w]) (Mettler Toledo, XS105) and added into the Oxygraph-2k respirometer containing MiR06 (37 °C), which makes it possible to increase the oxygen concentration in the chamber with hydrogen peroxide (H_2O_2). Between 1 and 3 µl of H_2O_2 (0.75% H_2O_2) was used in each experiment. Homogenate (2.0 ml) was added into the respirometer chamber.

Table 1
Subject characteristics.

Gender	3 females/6 males
Age (years)	58 ± 4
Weight (kg)	84 ± 3
Height (m)	1.74 ± 0.03
BMI (kg/m^2)	28 ± 1
Fat (%)	32 ± 3

Note. Data are presented as means ± standard errors. BMI, body mass index.

Table 2
Coefficients of variation between duplicate measurements (CI+II$_P$) from respiratory analysis of human skeletal muscle.

	Pfi (n = 9)	SH1 (n = 9)	SH2 (n = 4)	SH3 (n = 4)	SH4 (n = 5)
CV (%)	11.3 ± 4.2	4.8 ± 1.3	8.8 ± 4.2	4.4 ± 2.8	8.4 ± 3.2

Note. Data are presented as means ± standard errors. The protocols are described in Materials and methods.