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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.

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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 30 °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 2). 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

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.

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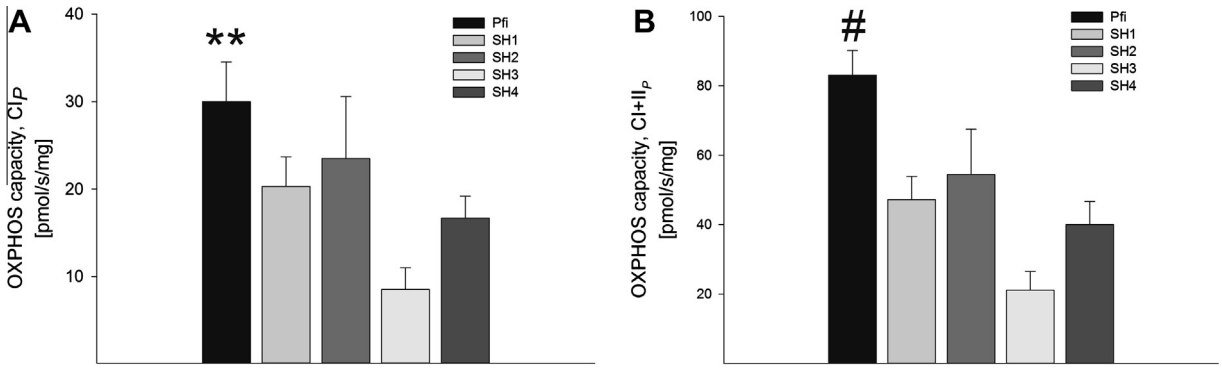

Fig.2. Effect of PBI shredder on mitochondrial respiratory capacity. (A) CI-linked active respiration (malate + glutamate + ADP) (CI_P). (B) CI+II-linked active respiration (malate + glutamate + ADP + succinate) ($CI+II_P$). Data are presented as means ± standard errors. $^{**}P < 0.05$ (Pfi vs. SH3); $^{\#}P < 0.05$) (Pfi vs. SH1, SH3, and SH4).

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.

Protocol

The protocol was conducted in a mitochondrial respiration buffer where catalase was added (MiR06) for both Pfi and shredder homogenate. The following substrates were added (final concentration): malate (2 mM) and glutamate (10 mM) to support electron entry to complex I (CI). This resting respiration mainly compensates for the proton leak (LEAK respiration, *L*) where no adenosine diphosphate (ADP) is present. Oxidative phosphorylation (OXPHOS) capacity (state 3 with saturating [ADP]) was reached by adding ADP (5 mM, CI_P). Succinate (10 mM) was added, stimulating CI+II-linked respiration and providing convergent electron input into the Q-junction simultaneously ($CI+II_P$) [17]. A maximal respiratory capacity is reached when these substrates are present in the respirometer chamber [17]. Cytochrome *c* (10 µM) was added to test the integrity of the outer mitochondrial membrane [2]. Electron transfer system capacity (ETS with CI+II-linked substrates, $CI+II_E$) was tested in a subgroup ($n = 4$ from Pfi, SH1, and SH4) of the human samples by titrating *p*-trifluoromethoxyphenylhydrazone (FCCP) (steps of 0.25 µM) until maximal noncoupled respiration was reached. Different ratios (substrate and coupling control ratios) can be calculated from the different titration steps obtained from the protocol used [17]. Mouse skeletal muscle was also investigated in a few samples ($n = 2$) to address species differences. The protocol used in human skeletal muscle was also applied in the animal experiments, but FCCP was not added because no excess capacity is seen in mice [17].

Statistics and calculations

Data are presented as means ± standard errors in the text and in all tables and figures. $P < 0.05$ was considered significant. Statistical analysis of differences among the five different preparations was carried out with a one-way analysis of variance. The restrictive assumptions, normality and equal variance, were checked before the statistical analysis was conducted. The Holm–Sidak post hoc test was used for further analysis.

The coefficient of variation (CV) between duplicate measurements was calculated using the following formula: CV (%) = 100 × (SD/mean), where SD is standard deviation.

Results

Mitochondrial OXPHOS capacity with CI-linked substrates (CI_P) was significantly ($P < 0.05$) decreased in SH3 compared with Pfi (Fig. 2A). Mitochondrial respiratory capacity with CI+II-linked substrates ($CI+II_P$) was significantly ($P < 0.05$) decreased in SH1, SH3, and SH4 compared with Pfi (Fig. 2B). No difference was seen in resting respiration (LEAK, state *L*) (malate + glutamate) between the different homogenates and Pfi (data not shown). Maximal noncoupled respiration was significantly ($P < 0.05$) higher in Pfi compared with SH1 and SH4 (data not shown). The cytochrome *c* response was significantly ($P < 0.05$) larger in SH1, SH2, SH3, and SH4 compared with Pfi, with no differences among SH1, SH2, SH3, and SH4 (Fig. 3A). The substrate control ratio for succinate ($CI_P/CI+II_P$) was comparable among all of the protocols (Fig. 3B). The RCR (*P*/*L* with CI-linked substrates) was significantly ($P < 0.05$) reduced in SH1, SH2, SH3, and SH4 compared with Pfi (Fig. 3C).

Furthermore, RCR was significantly ($P < 0.05$) lower in SH3 compared with SH1, SH2, and SH4 (Fig. 3C). The uncoupling control ratio (*P*/*E* in the CI+II substrate state) was significantly ($P < 0.05$) lower in SH1 and SH4 compared with Pfi, with no difference between SH1 and SH4 (Fig. 3D).

The flux control ratio (*L*/*E*: $CI_L/CI+II_E$), combining the effect of coupling and substrate control, was significantly higher in SH1 and SH4 compared with Pfi (data not shown).

The CVs between the two duplicate measurements in the five different preparations are shown in Table 3.

There was an equal loss of muscle tissue among the four different protocols, so that the amounts of muscle tissue that were actually shredded were similar in SH1, SH2, SH3, and SH4 at 67 ± 2, 74 ± 6, 66 ± 3, and 75 ± 5%, respectively (Table 3).

Mitochondrial respiratory capacity in mouse skeletal muscle with CI (CI_P), as well as CI+II-linked substrates ($CI+II_P$), was reduced in SH1 and SH4 compared with Pfi (Table 4). A higher cytochrome *c* response was seen after homogenization (SH1 and SH4) in mouse skeletal muscle (Table 4).

Discussion

The major and novel finding in the current study is that maximal mitochondrial respiratory capacity is reduced in a human muscle homogenate obtained by the shredder methodology compared with permeabilized muscle fibers (Fig. 1A and B). The lower respiratory fluxes were accompanied by increased cytochrome *c* response in the different prepared homogenates compared with Pfi (Fig. 3A), indicating a loss of the integrity of the outer mitochondrial membrane. However, within the individual tissue preparation, the response to adding a substrate (e.g., succinate) was maintained, a conclusion supported by the similar succinate substrate control ratio between Pfi and shredded homogenates (Fig. 3C).

One study tested whether using a shredder for homogenization of mitochondria provides intact and functional isolated mitochondria [22]. This study was conducted in rat skeletal muscle and re-

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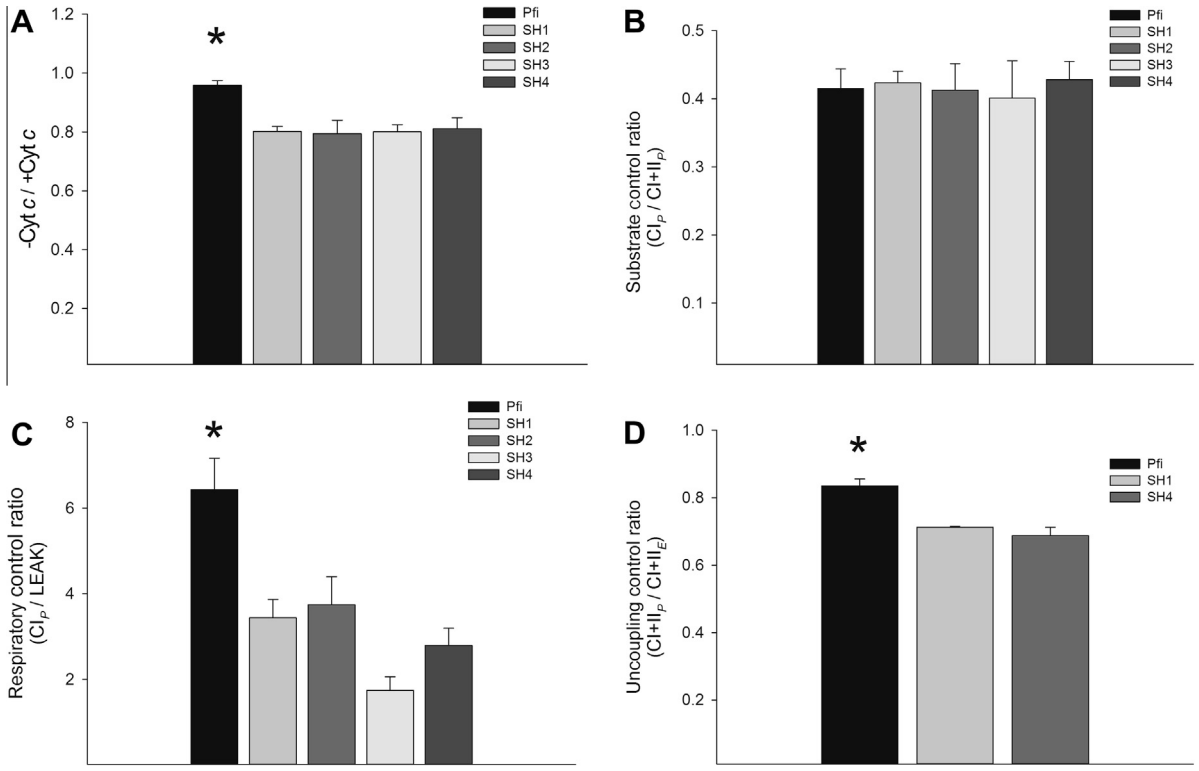

Fig.3. Effect of PBI shredder on different mitochondrial ratios. (A) Ratios among respiratory capacity, CI+II$_P$ (no cytochrome *c*), and after adding cytochrome *c*. If the ratio is close to 1, it means no effect of cytochrome *c*, indicating intact outer mitochondrial membranes. Cyt *c*, cytochrome *c*. (B) Effect of adding succinate, that is, substrate control ratio calculated as (respiratory capacity, CI$_P$/respiratory capacity, CI+II$_P$). (C) Respiratory adenylate control ratio calculated as (CI$_P$/CI$_L$). (D) Uncoupling control ratio (*P*/*E* = CI+II$_P$/CI+II$_E$) in a subgroup of samples (only Pfi, SH1, and SH4). Data are presented as means ± standard errors. $^*P < 0.05$ (Pfi vs. other protocols).

Table 3
Percentages of initial human skeletal muscle weight that are actually shredded.

	Initial weight (mg)	Remaining after shredding (mg)	%Shredded
SH1	7.75 ± 0.79	2.54 ± 0.25	67 ± 2
SH2	7.66 ± 0.48	1.92 ± 0.42	74 ± 6
SH3	5.94 ± 0.63	1.96 ± 0.09	66 ± 3
SH4	8.59 ± 0.75	2.08 ± 0.32	75 ± 5

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

Table 4
Mitochondrial respiratory capacities of mouse skeletal muscle (soleus) using the SH1 and SH4 protocol compared with Pfi.

	Pfi	SH1	SH4
CI$_P$ (pmol/s/mg)	38 ± 1	10 ± 1	12 ± 10
CI+II$_P$ (pmol/s/mg)	60 ± 1	20 ± 1	22 ± 12
−Cyt *c*/+Cyt *c*	0.88 ± 0.12	0.67 ± 0.01	0.58 ± 0.17
SCR (CI+II$_P$/CI$_P$)	1.59 ± 0.01	1.98 ± 0.17	3.92 ± 2.35
RCR (OXPHOS/LEAK)	5.28 ± 0.04	20.87 ± 17.92	1.16 ± 2.18

Note. Data are presented as means ± SEM. No statistics are provided on the animal samples ($n = 2$). The protocols are described in Materials and methods. Cyt *c*, cytochrome *c*; SCR, substrate control ratio.

ported that functional mitochondria were obtained after homogenization and differential centrifugation. No absolute respiratory rates were provided in this article, and the addition of cytochrome *c* is not mentioned, which otherwise would have provided an indication of the intactness of the mitochondria [22]. In the study by Gross and coworkers, an electron microscopy (EM) image is shown, where some disrupted mitochondria can be seen after the homogenization procedure [22]. The elevated cytochrome *c* response in the shredded samples in the current study is a functional test of the intactness of the mitochondria, and thus the data are compatible with the disrupted mitochondria in the EM images from Gross and coworkers [22]. The RCR was significantly reduced in the different homogenates compared with Pfi in the current study using the entire tissue homogenate, in contrast to further isolating mitochondria and potentially separating intact and broken mitochondria [22]. The differences in RCR in the current study are due to a reduced CI-linked coupled respiratory capacity (CI$_P$) because LEAK respiratory rates were similar across the tissue preparations. This indicates that impairment is seen in the homogenized samples only when respiration is coupled to phosphorylation; that is, the OXPHOS capacity seems to be impaired by the shredding procedure. Furthermore, the uncoupling control ratio found in the current study indicates a higher degree of electron transport chain reserve capacity in the homogenates compared with the Pfi [17]. It should be mentioned that the numerical mitochondrial respiratory flux rate from the Pfi in the current study corresponds with previous findings [1,5,10,25].

The CV between the different homogenate protocols and the Pfi was not different, although the Pfi had a higher (but not significant) CV as expected, which indicates that the technical variation is higher when Pfi are used. Thus, the shredding procedure seems to serve the purpose of minimizing the interpersonal variation.

When skeletal muscle from mice were investigated, the same pattern was seen—a reduced mitochondrial respiratory capacity with CI (CI$_P$) and CI+II (CI+II$_P$)-linked substrates present in the homogenized samples compared with the Pfi—and this was accompanied by an increased cytochrome *c* response in the homogenized samples. The mitochondrial respiratory rates from Pfi in the current study correspond with previously published literature from mice skeletal muscle [26,27].

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In conclusion, the PBI shredder technique does not produce functionally intact mitochondria in skeletal muscle. A reduced maximal OXPHOS capacity is seen after the shredder homogenization compared with Pfi. This is accompanied by increased respiratory rates after adding cytochrome *c* into the different homogenates and a reduced RCR. The differences in functional tests between the sample preparation techniques (shredding or Pfi) were seen in skeletal muscle of different mammalian species. A high degree of human manual skills is still needed in the preparation and permeabilization of skeletal muscle fibers used for respiratory analysis.

Our results on the comparison of permeabilized fibers versus shredder homogenate of skeletal muscle are in contrast to reports on shredder homogenate of mouse heart, brain, and liver; the cytochrome *c* effect was zero or <5%, mitochondrial quality was preserved in the tissue homogenate compared with permeabilized fibers (heart) or isolated mitochondria (brain), and respiratory capacity was retained in tissue homogenate compared with Pfi [24]. These differences reflect the tissue structure and illustrate the importance of optimization and critical evaluation of techniques used in mitochondrial physiology.

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