Functional muscle impairment in facioscapulohumeral muscular dystrophy is correlated with oxidative stress and mitochondrial dysfunction


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Facioscapulohumeral muscular dystrophy (FSHD), the most frequent muscular dystrophy, is an autosomal dominant disease. In most individuals with FSHD, symptoms are restricted to muscles of the face, arms, legs, and trunk. FSHD is genetically linked to contractions of the D4Z4 repeat array causing activation of several genes. One of these maps in the repeat itself and expresses the DUX4 (the double homeobox 4) transcription factor causing a gene deregulation cascade. In addition, analyses of the RNA or protein expression profiles in muscle have indicated deregulations in the oxidative stress response. Since oxidative stress affects peripheral muscle function, we investigated mitochondrial function and oxidative stress in skeletal muscle biopsies and blood samples from patients with FSHD and age-matched healthy controls, and evaluated their association with physical performances. We show that specifically, oxidative stress (lipid peroxidation and protein carbonylation), oxidative damage (lipofuscin accumulation), and antioxidant enzymes (catalase, copper–zinc-dependent superoxide dismutase, and glutathione reductase) were higher in FSHD than in control muscles. FSHD muscles also presented abnormal mitochondrial function (decreased cytochrome c oxidase activity and reduced ATP synthesis). In addition, the ratio between reduced (GSH) and oxidized glutathione (GSSG) was strongly decreased in all FSHD blood samples as a consequence of GSSG accumulation. Patients with FSHD also had reduced systemic antioxidative response molecules, such as low levels of zinc (a SOD cofactor), selenium (a GPx cofactor involved in the elimination of lipid peroxides), and vitamin C. Half of them had a low ratio of gamma/alpha tocopherol and higher ferritin concentrations. Both systemic oxidative stress and mitochondrial dysfunction were correlated with functional muscle impairment. Mitochondrial ATP production was significantly correlated with both quadriceps endurance (TlimQ) and maximal voluntary contraction (MVCQ) values (rho = 0.79, P = 0.003; rho = 0.62, P = 0.05, respectively). The plasma concentration of oxidized glutathione was negatively correlated with the TlimQ, MVCQ values, and the 2-min walk distance (MWT) values (rho = −0.60, P = 0.03; rho = −0.56, P = 0.04; rho = −0.93, P < 0.0001, respectively). Our data characterized oxidative stress in patients with FSHD and demonstrated a correlation with their peripheral skeletal muscle dysfunction. They suggest that antioxidants that might modulate or delay oxidative insult may be useful in maintaining FSHD muscle functions.

Keywords:
Facioscapulohumeral muscular dystrophy (FSHD)
4-Hydroxy-2-nonenal
Thiobarbituric acid-reactive substances
Lipofuscin
Oxidative stress
Protein oxidation
Radical oxygen species
Mitochondria dysfunction
Antioxidants

**Abstract**

Facioscapulohumeral muscular dystrophy (FSHD), the most frequent muscular dystrophy, is an autosomal dominant disease. In most individuals with FSHD, symptoms are restricted to muscles of the face, arms, legs, and trunk. FSHD is genetically linked to contractions of the D4Z4 repeat array causing activation of several genes. One of these maps in the repeat itself and expresses the DUX4 (the double homeobox 4) transcription factor causing a gene deregulation cascade. In addition, analyses of the RNA or protein expression profiles in muscle have indicated deregulations in the oxidative stress response. Since oxidative stress affects peripheral muscle function, we investigated mitochondrial function and oxidative stress in skeletal muscle biopsies and blood samples from patients with FSHD and age-matched healthy controls, and evaluated their association with physical performances. We show that specifically, oxidative stress (lipid peroxidation and protein carbonylation), oxidative damage (lipofuscin accumulation), and antioxidant enzymes (catalase, copper–zinc-dependent superoxide dismutase, and glutathione reductase) were higher in FSHD than in control muscles. FSHD muscles also presented abnormal mitochondrial function (decreased cytochrome c oxidase activity and reduced ATP synthesis). In addition, the ratio between reduced (GSH) and oxidized glutathione (GSSG) was strongly decreased in all FSHD blood samples as a consequence of GSSG accumulation. Patients with FSHD also had reduced systemic antioxidative response molecules, such as low levels of zinc (a SOD cofactor), selenium (a GPx cofactor involved in the elimination of lipid peroxides), and vitamin C. Half of them had a low ratio of gamma/alpha tocopherol and higher ferritin concentrations. Both systemic oxidative stress and mitochondrial dysfunction were correlated with functional muscle impairment. Mitochondrial ATP production was significantly correlated with both quadriceps endurance (TlimQ) and maximal voluntary contraction (MVCQ) values (rho = 0.79, P = 0.003; rho = 0.62, P = 0.05, respectively). The plasma concentration of oxidized glutathione was negatively correlated with the TlimQ, MVCQ values, and the 2-min walk distance (MWT) values (rho = −0.60, P = 0.03; rho = −0.56, P = 0.04; rho = −0.93, P < 0.0001, respectively). Our data characterized oxidative stress in patients with FSHD and demonstrated a correlation with their peripheral skeletal muscle dysfunction. They suggest that antioxidants that might modulate or delay oxidative insult may be useful in maintaining FSHD muscle functions.

**Abbreviations:** FSHD, facioscapulohumeral muscular dystrophy; DUX4, double homeobox 4 gene; ROS, reactive oxygen species; HNE, 4-hydroxy-2-nonenal; TBARS, thiobarbituric acid-reactive substances; MnSOD, manganese-dependent superoxide dismutase; Cu-SOD, copper–zinc-dependent superoxide dismutase; GSH, reduced glutathione; GSSG, oxidized glutathione; MVC, maximal voluntary contraction; TlimQ, endurance limit time; 2-MWT, 2 minute walking test

*Correspondence to: INSERM, U1046, Bâtiment Crastes de Paulet, 371 avenue du Doyen Giraud, 34 295 Montpellier, France. Fax: +33 0 4 67 41 52 21.
E-mail address: dalila.laoudj-chenivesse@inserm.fr (D. Laoudj-Chenivesse).
**Introduction**

Facioscapulohumeral muscular dystrophy (FSHD) is an autosomal dominant muscle disease characterized by progressive weakness and atrophy of facial, shoulder girdle, and upper arm muscles. Magnetic resonance imaging also revealed widespread involvement of leg muscles, particularly of the tibialis anterior and medial gastrocnemius [1], that may result in walking difficulties. Approximately 10% of all patients and 20% of those who are older than 50 years will eventually become wheelchair dependent for outdoor activities [2,3]. Less frequent clinical manifestations including respiratory [4] and cardiac conduction [5] defects may occur in patients severely affected with FSHD. Finally, many patients complain of pain and fatigue, which are likely to reduce the levels of daily activity.

FSHD is genetically linked to deletions in chromosome 4q35 [6,7] within an array of D4Z4 repeated elements with each composing the double homeobox 4 (DUOX) gene [8,9]. Several analyses of gene expression in FSHD muscle have produced partially overlapping and sometimes contradictory results [10–15]. D4Z4 contractions are proposed to cause epigenetic changes, which ultimately increase expression of genes with myopathic potential [6,16–19], particularly ANT1, FRG1, DUX4c, FRG2, and DUX4 [9,20–24]. Expression of the DUX4 protein in muscles is now considered the major contributor to the molecular pathogenesis of FSHD [23,25,26]. DUX4 is a transcription factor that initiates a large gene deregulation cascade. Its forced expression in muscle cells is toxic and induces typical FSHD molecular changes [9,25–27]. Expression in muscle cells is toxic and induces typical FSHD molecular features, such as muscle atrophy markers and TP53, diminished myogenic differentiation capacity, sensitivity to oxidative stress, and genes expressed in germline and early stem cells where DUX4 is normally expressed [22,24,25,27,28].

In addition, analysis of the RNA or protein expression profiles in peripheral muscles of patients with FSHD compared to healthy controls revealed that many genes differentially expressed in affected and nonaffected FSHD muscles are specifically involved in oxidative stress responses [14,29–33]. Indeed, these genes are not deregulated in other muscular dystrophies such as DMD and LGMD2B and the activation of some of them (ANT1 encoding a mitochondrial protein; DUOX4 encoding a transcription factor) are linked to the chromatrin opening in 4q35, suggesting that aberrations in this pathway could be the primary events rather than consequences of the muscle pathology [29–31]. The FSHD specificity of this defect is also underscored by the susceptibility of primary myoblasts to oxidative agents and alteration in gene expression and protein synthesis involved in oxidative stress responses [14,32].

Since oxidative stress affects peripheral muscle function, we investigated mitochondrial function and oxidative stress in blood samples and skeletal muscle biopsies from patients with FSHD and healthy controls. We then evaluated their association with physical performance.

**Materials and methods**

**Patients**

Fifteen patients with FSHD (9 males and 6 females) and 9 healthy subjects (6 males and 3 females) matched for age (FSHD, 38.9 years ± 10.8; controls, 35.7 years ± 10.2) and physical activity level were sequentially recruited over 2 years in the Department of Clinical Physiology, University Hospital, Montpellier (France). Patients with FSHD were not wheelchair bound and diagnosis was based on clinical examination, number of D4Z4 units (4 to 9 by DNA analysis), and positive family history for FSHD. The Brooke and Vignos functional scales are used to grade arm and leg function, respectively [34]. Twelve of 15 patients were ranked between 1 and 2 for arms and in rank 1 for legs according to that scale. The other three patients were more affected and ranked between 4 and 5 for arms and for legs. Controls and patients were sedentary (Voorrips questionnaire’s score < 9.4) [35] and did not participate in any kind of physical training or rehabilitation. All were nonsmokers, had no comorbidity (such as cardiac or pulmonary disease, diabetes or human immunodeficiency virus), and were not taking any drug, including vitamins and/or antioxidants. The protocol was approved by the local review board (Ethics Committee of the CHU Saint Eloi, Montpellier, France; reference number 05 05 03). Participants received extensive information about the study before providing their written informed consent. All functional and clinical evaluations and muscle biopsies were performed in the Department of Clinical Physiology, University Hospital, Montpellier (France).

**Clinical and functional evaluation**

Spirometry was performed using a plethysmograph (Spirometer Vmax, Sensormedics, US). Maximal inspiratory (Pmax) and expiratory (Pimax) pressures were also measured [36].

For maximal voluntary contraction and endurance of quadriceps and deltoid muscles, each leg’s quadriceps maximal voluntary contraction (MVCq) and endurance (Tlimq) were assessed on an adapted exercise bench (Kettler, Germany) in seated position with knees and hips flexed at 90°, as previously reported [37–40]. MVCq was recorded through a strain gauge linked to a computer interface (Biopac, Acknowledge, France). Three to five maximal trials were performed to obtain at least two values with less than 10% variability. The best value was taken as the MVCq.

To assess quadriceps endurance (Tlimq), subjects had to maximally extend each knee against a weight that corresponded to 30% of the MVCq at a pace of 10 movements per minute, imposed by an audio signal, until exhaustion. The duration of the endurance test (in seconds) was called quadriceps endurance limit time (Tlimq). The leg with the highest Tlimq was considered the “reference leg” and chosen for the biopsy.

The deltoid maximal isometric voluntary contraction (MVCp) was bilaterally assessed using the same signal recorder as quadriceps measurements with the strain gauge fixed in order to maintain shoulder and arm at 90°. The subjects were in the sitting position and were asked to develop a maximal contraction by elevating the arm against the fixed strain gauge, until exhaustion. The duration of the endurance test (in seconds) was called quadriceps endurance limit time (Tlimq).

The 2-minute walking test (2-MWT) was carried out as previously described [41,42]. Subjects were asked to walk back and forth around two cones placed in an indoor, straight, 30-m corridor for 2 min. Two tests separated by 60 min were performed at a maximum walking pace with the goal being to cover as much distance as possible and the longest distance covered during the 2-MWT was retained.

Magnetic resonance imaging (MRI) was performed on 9 patients with FSHD by using a 1.5-T unit (Magnetom Avento, Siemens, Erlangen, Germany). Transverse T1-, T2-weighted, and inversion recovery images were taken. No contrast agent was used. Transverse T1-weighted and T2-weighted images served for anatomical localization of muscles. The analysis of extent of muscle involvement and muscle atrophy was performed by an expert radiologist (A.T.). In all images, the lumbrical muscle of the hand and the tibialis anterior muscle were used as normal controls. During the 1.5-T MRI, images were reconstructed in sagittal and coronal planes.

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recovery images were obtained on both thighs. No contrast media were administered.

**Muscle biopsies**

Samples from the quadriceps muscle of the “reference legs” were obtained by needle biopsy after local lidocaine anesthesia at mid-thigh level, in the vastus lateralis, using the Bergström technique [43,44]. Each biopsy (approximately 300 mg) was divided in three portions (see supplementary data): one part (100 mg) to evaluate the respiratory parameters and mitochondrial adenosine triphosphate (ATP) synthesis; another part (40 mg) to evaluate tissue organization by hematoxylin/eosin staining of cross sections and to investigate mitochondrial morphology by electron microscopy; the last part was immediately frozen in isopentane cooled to the freezing point with liquid nitrogen and stored at −80 °C, for analysis of oxidative stress markers. In 12/15 patients weakly affected by FSHD (Brooke-Vignos functional scale), analysis of the histological tissue organization and MRI showed no fibrosis. However, in the 3 more affected patients, muscle biopsies showed histological signs of severe end-stage feature of dystrophic process, and were too affected to allow any analysis, in agreement with no observable vastus lateralis muscle structure in MRI images (data not shown).

**Blood and urine samples**

Concomitantly with the muscle biopsy, venous blood samples were collected to determine the serum levels of oxidative stress and inflammatory markers. In two patients with FSHD, (different from the three patients where biopsy samples were too affected to allow any analysis), it was not possible to obtain enough venous blood samples to determine all the parameters. Each parameter was routinely determined at the CHU Clinical Laboratories of the University of Liège, Belgium. Normal reference values in a population of 100 healthy, 18- to 60-year-old individuals have recently been published [45–47]. Samples were collected in ethylenediaminetetraacetic acid (EDTA), sodium–heparin as anticoagulant, or clot-activating gel according to the investigated parameter and immediately centrifuged; plasmas were frozen in dry ice and kept at −80 °C until analysis (see supplemental data). Urine samples were collected to determine the level of an oxidative damage DNA adduct and kept at −80 °C until analysis (see supplemental data).

**Statistical analysis**

Quantitative parameters are presented as means ± standard deviation (SD) or standard error of the mean (SEM). Their distribution was compared with the Student t test (a transformation for a normalized distribution was applied when necessary) or the Mann-Whitney test. The distribution of inflammatory parameters was compared with the two-sided Fisher’s exact test. Correlations were fitted with the Spearman rank sum test because linearity could not be assumed for all correlations. For all analyses, α was set at 5%. Statistical analyses were performed with the Stata v6.0 software (Stata Corporation, College Station, TX).

**Results**

**Functional limitations in patients with FSHD**

Spirometric values did not show any significant respiratory difference between patients with FSHD and controls, except for the maximal expiratory pressure (P_{Emax}), which was significantly lower in patients (Table 1). They also had lower maximal voluntary contraction (MVC) and endurance time limit (T_{Lim}) for both quadriceps and deltoid than controls (Table 1). However, the endurance time limit decrease in quadriceps (T_{LimQ}) was not significant (P = 0.10). Moreover, in patients there was a significant positive relationship between the T_{Lim} and the MVC values (rho = 0.65; P = 0.01) in quadriceps, but not in deltoid. Exercise tolerance, assessed with the 2-MWT, was significantly lower in patients with FSHD than in controls (Table 1).

**Oxidative stress in FSHD muscles**

Lipofuscin inclusions, a marker of cumulative oxidative stress [48–51], were significantly more abundant in FSHD than in control quadriceps biopsies (P<0.001) (Fig. 1A). They were mostly located in the subsarcolemmal area, a region rich in mitochondria and an important source of reactive oxygen species (ROS) (Fig. 1B, inset). The level of 4-hydroxy-2-nonenal (HNE) and the concentration of thiobarbituric acid-reactive substances (TBARs), two major end products of lipid peroxidation, as well as the level of protein carbonylation, which is the most widely used marker of oxidative stress [52,53], were all significantly higher in FSHD than in control muscles (P<0.001 for the three) (Fig. 2A, B, and C).

While the decrease of MnSOD in FSHD muscles was not significant (P = 0.22) (data not shown), catalase, copper–zinc-dependent superoxide dismutase (Cu-Zn SOD), and glutathione reductase (GR) (all intracellular antioxidant enzymes) were significantly more elevated in FSHD than control muscles (P = 0.05, P = 0.0005, and P = 0.0006, respectively) (Fig. 2D).

Together, these results demonstrate that an increase in oxidative stress in FSHD skeletal muscle biopsies is associated with altered expression of antioxidant enzymes.

### Table 1

<table>
<thead>
<tr>
<th>Spirometric and musculoskeletal functional data in 15 patients with FSHD and 9 controls.</th>
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<tbody>
<tr>
<td><strong>FSHD patients (n=15) Mean (SD)</strong></td>
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<tr>
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<tr>
<td><strong>Vital capacity</strong></td>
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<tr>
<td><strong>Total lung capacity</strong></td>
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<tr>
<td><strong>Maximal inspiratory pressure</strong></td>
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<td><strong>Maximal expiratory pressure</strong></td>
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<tr>
<td><strong>Peripheral muscle function</strong></td>
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<tr>
<td><strong>Maximal contraction (MV{C}Q): best leg (kg)</strong></td>
</tr>
<tr>
<td><strong>Endurance (T{Lim}Q): best legs (seconds)</strong></td>
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<tr>
<td><strong>Maximal contraction (MV{C}D): dominant arm (kg)</strong></td>
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<tr>
<td><strong>Endurance (T{Lim}D): dominant arm (seconds)</strong></td>
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<tr>
<td><strong>2-minute walking test (meters)</strong></td>
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</table>

Data are mean ± standard deviation (SD).
Fig. 1. Quantification and localization of lipofuscin inclusions in vastus lateralis muscle biopsies from 12 patients with FSHD and 9 controls (CON). (A) Quantification of lipofuscin inclusions (number of inclusions/fiber). Data are means ± standard error of the mean (SEM). *** P < 0.0005. (B) Localization of lipofuscin inclusions. Representative image of dystrophin membrane staining (red), autofluorescent lipofuscin granules (yellow), and merged with controls (top panel) and patients with FSHD (bottom panel). Bar scale: 50 μm.

Fig. 2. Oxidative stress and antioxidant response in quadriceps muscle biopsies from 12 patients with FSHD and 9 controls (CON). Data are means ± standard error of the mean (SEM). * P < 0.05; ** P < 0.005. (A) Level of 4-hydroxynonenal (HNE)-modified proteins (arbitrary units, a.u.). (B) Concentration of thiobarbituric acid-reactive substances (TBARs) (nM/mg protein). (C) Level of protein carbonylation, the most widely used marker of oxidative stress (arbitrary units, a.u.). (D) Expression of catalase, copper–zinc-dependent SOD (Cu-Zn SOD), and glutathione reductase (GR) (arbitrary units, a.u.).
Alteration of mitochondrial ultrastructure in the FSHD skeletal muscles

The quadriceps ultrastructure and particularly the mitochondrial organization were analyzed in longitudinal sections. In control muscles (Fig. 3A) intermyofibrillar mitochondria (arrowhead) were mainly organized in pairs at the I-band, as described [54]. In FSHD muscle fibers (Fig. 3B, C, and D), a similar mitochondrial organization was observed; however, in some areas the myofibril and mitochondria ultrastructure was markedly altered, e.g., large mitochondria pools in the intermyofibrillar (arrowhead) and subsarcolemmal (arrow) compartments (Fig. 3B). In addition, within individual muscle fibers, regions containing normal myofibrils alternated with areas of pronounced myofibrillar disorganization often associated with mitochondria accumulations (Fig. 3C). This abnormal proliferation of intermyofibrillar mitochondria was associated with accumulation of “granulofilamentous material” (Fig. 3C, arrow). At higher magnification, mitochondria with some badly formed cristae and apparent swelling (Fig. 3D, arrow) or separation of the inner and outer membranes were observed in these disorganized regions.

Mitochondrial dysfunction in FSHD skeletal muscles

Mitochondrial respiration was studied in situ in saponin-skinned quadriceps fibers as described [55,56] (Table 2). While the maximal ADP-stimulated respiration (state 3; \( V_{\text{max}} \)) with complex I (pyruvate-malate) and complex II (succinate/rotenone) substrates was comparable in FSHD and control muscle fibers, a significant decrease in cytochrome c oxidase (COX) activity (the last respiratory chain enzyme), determined with complex IV activators (\( N,N,N,N'-\text{tetramethyl-p-phenylenediamine (TMPD) and ascorbate} \)), was observed. However, the reduction of COX activity did not affect the total mitochondrial respiration rates evaluated with different substrates that assess electron transport in the respiratory chain. The Respiratory Control Index (RCI) was comparable in FSHD and control skinned fibers as well as the level of citrate synthase (an enzymatic marker of the mitochondrial matrix). The altered complex IV activity did not result from decreased cytochrome c content because similar levels were found in FSHD and control muscles (Table 2).

The ATP synthesis capacity of the OXPHOS pathway was also measured directly in saponin-skinned fibers suspended in mitochondrial medium and supplied with respiratory substrates and ADP [57], as in the oxygraphic experiments. A decrease in ATP synthesis and in the ATP/O values (ratio between ATP synthesis and oxygen consumption) was observed in FSHD fibers, using pyruvate+malate or succinate as substrates and TMPD/ascorbate as activators. However, ATP synthase (ATPase) concentration, assessed by immunodetection on Western blots with antibodies against its alpha or beta subunits, was comparable in FSHD and control samples (Table 2). A negative correlation was only found between the mitochondrial ATP synthesis and the level of protein carbonylation (\( \rho = -0.65; P = 0.03 \)).

In conclusion both ultrastructural morphological changes and functional assays point to deregulations of the mitochondrial respiratory machinery in FSHD muscles.

Fig. 3. Evaluation of mitochondrial morphology in quadriceps muscle biopsies from 12 patients with FSHD and 9 controls by transmission electron microscopy. Longitudinal sections from control (A) and FSHD (B, C, and D) quadriceps muscle. Bar scale: 1 \( \mu \text{M} \).
Systemic oxidative stress and inflammation in FSHD

For all systemic oxidative stress markers analyzed in this study, the mean values remained within the reference norm established by the Clinical Laboratories of the University of Liege Hospital [47], in the control group, while differences were observed in the disease group (Fig. 4). Moreover, this analysis indicated that patients with FSHD had higher oxidative damage than controls. Specifically, patients had lower levels of oxidized DNA and lipid peroxidation compared to the control group (P=0.09 and P=0.002, respectively) (Fig. 4). However, the oxidized DNA decrease in blood was not significant. The ratio between reduced (GSH) and oxidized glutathione (GSSG) was strongly decreased in all FSHD blood samples compared to the control group (P<0.001) as a consequence of GSSG accumulation (P<0.001), while the GSH value remained normal (Fig. 4).

Although no significant differences were observed between FSHD and control samples for many antioxidative stress molecules, such as vitamin A, ubiquinone, β-carotene, superoxide dismutase (SOD), glutathione peroxidase (GPx), and myeloperoxidase,
Table 3
Association of both mitochondrial dysfunction and oxidative stress and inflammation with functional parameters in patients with FSHD.

<table>
<thead>
<tr>
<th>Mitochondrial ATP production</th>
<th>MVCQ</th>
<th>2-MWT</th>
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<tbody>
<tr>
<td>n = 12</td>
<td></td>
<td></td>
</tr>
<tr>
<td>rho = 0.79</td>
<td>rho = 0.62</td>
<td>-</td>
</tr>
<tr>
<td>P = 0.003</td>
<td>P = 0.05</td>
<td></td>
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<tr>
<td>n = 13</td>
<td></td>
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</tr>
<tr>
<td>rho = 0.58</td>
<td>rho = 0.74</td>
<td>-</td>
</tr>
<tr>
<td>P = 0.03</td>
<td>P = 0.003</td>
<td></td>
</tr>
<tr>
<td>rho = 0.60</td>
<td>rho = 0.56</td>
<td>rho = 0.93</td>
</tr>
<tr>
<td>P = 0.03</td>
<td>P = 0.04</td>
<td>P = 0.0001</td>
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</table>

Spearman's rank correlation coefficient, rho. Underline: moderate correlations between 0.56 and 0.62.

Discussion

This study shows that, in patients with FSHD, functional muscle alterations are associated with mitochondrial dysfunction and oxidative stress imbalance, further supporting the idea that oxidative stress might play a role in FSHD pathophysiological mechanisms [14,29–31].

The biochemical evidence of mitochondrial dysfunction was provided by a decrease in COX activity without any effect on total mitochondrial respiration. The V_{max} (state 3) with pyruvate-malate was not significantly altered in FSHD patients, suggesting that COX may be inhibited without any effect on mitochondrial respiration up to a threshold beyond which a further decline in COX activity might exert an inhibitory effect. COX activity decline represents a focal electron transport chain deficit in a nonclinically affected tissue (vastus lateralis) and a decline in the ROS detoxification capacity of muscle cells. A modest reduction of COX activity can lead to ROS production [58] that can further reduce COX activity and decrease ATP levels due to oxidative damage to other mitochondrial components. Moreover, the significant reduction in COX activity may partially explain the impaired ability to synthesize ATP in FSHD mitochondria.

Since coupling of electron transport to ATP synthesis (indicated by the RCI ratio) did not differ between FSHD and control muscle fibers (Table 2), the reduced ATP/O ratio suggests that the alteration of oxidative phosphorylation (OXPHOS pathway) occurs in the PHOS part, i.e., the ATP synthesis rate. Impaired energy metabolism resulting from mitochondrial dysfunction has also been proposed to render cells more vulnerable to "cellular stressors" due to changes in the energy-dependent cell membrane potential [59]. These functional disturbances were associated with striking mitochondrial morphological changes in FSHD quadriceps, as revealed by electron microscopy. Both subsarcolemmal and intramyofibrillar mitochondria were affected, which indicated common alterations independent of the subcellular localization.

Furthermore, the data showing a negative correlation between mitochondrial ATP synthesis and protein carbonylation suggest that oxidative stress in skeletal muscle could probably be one of the major determinants of mitochondrial alterations. However, we cannot exclude that mitochondrial disruption occurs for a reason other than oxidative stress and that accumulation of protein carbonyls represents an effect of mitochondrial dysregulation.

These alterations were also associated with a significant increase of Cu-Zn SOD, catalase, and GR concentrations. The reasons for these defects in FSHD muscle are unknown, but cannot be considered the consequence of tissue degeneration. Indeed, no prominent muscle wasting could be observed in the vastus lateralis biopsies from this group of patients with FSHD, either by histologic analysis or by MRIs (Fig. 5). However, it remains unclear whether the decline in mitochondrial respiratory function in FSHD muscle results from oxidative stress alone or is the consequence of synergistic effects of multiple factors, acting either independently or cooperatively with oxidative stress. Among several genes that have been proposed to be involved in the FSHD pathophysiology based on their up-regulation in patient-derived tissues or cell lines [9,20–22,24] some are connected to oxidative stress such as the mitochondrial ADP/ATP carrier ANT1 [30]. Similarly, although the precise function of the FRG1 gene product is not known, overexpression studies in different animal models support a role in RNA processing [60,61], in actin bundling, and in the regulation of muscle and vasculature development [11,62,63]. The controversy in the literature about the actual expression levels of these genes in FSHD muscles reviewed in [64] might reflect the typical heterogeneity of the patients could present significantly lower levels of zinc (a SOD cofactor) and selenium (a GPx cofactor involved in the elimination of lipid peroxides) as compared to the controls (P < 0.001 and P < 0.003, respectively) (Fig. 4). Decreased plasma levels of vitamin C (P = 0.006) (Fig. 4) and of its ascorbate precursor (FSHD: 1.36 ± 0.30 a.u.; controls: 2.73 ± 1.47 a.u.; P = 0.04) were also observed in patients with FSHD as well as a lower ratio of gamma/alpha tocopherol and higher ferritin concentrations than in controls (P < 0.001 and P = 0.004, respectively) (Fig. 4). These results suggest that patients with FSHD also have a reduced systemic antioxidative response. Except for GSSG that was negatively correlated with the GSH/GSSG ratio (rho = 0.90, P < 0.0001), no correlation was observed between any measures of injury nor between antioxidant stress molecules.

Increased expression of the proinflammatory cytokines RANTES (regulated on activation, normal T-cell expressed and secreted), TNF-α (tumor necrosis factor-alpha), and IFN-α (interferon alpha-2) was found in all patients with FSHD (P < 0.001 vs controls). MCP1 (monocyte chemotactic protein-1) and IL6 (interleukin 6) were increased in 67% of subjects with FSHD (P = 0.001 and P = 0.01, respectively, vs controls). IL1-α (interleukin 1-alpha) was increased in 40% of them (P = 0.03) and IL1-β (interleukin 1-beta) and IFN γ (interferon gamma) in 33% of them (P = 0.03 and P = 0.05, respectively) (Supplemental Fig. 1s). A positive correlation was only found between TNF-α and GSSG levels (rho = 0.62; P = 0.02).

Association of both mitochondrial dysfunction and oxidative stress and inflammation with functional parameters in patients with FSHD.

These data indicate that patients with FSHD have abnormal mitochondrial function and increased oxidative stress and inflammatory response in comparison with controls. Both systemic oxidative stress and mitochondrial dysfunction were correlated with functional muscle impairment. The correlations observed in the disease group were significant but some of them were moderate (Table 3). Mitochondrial ATP production was significantly correlated with both quadriceps endurance (T_{limQ}) and maximal voluntary contraction (MVCQ) values (rho = 0.79, P = 0.003; rho = 0.62, P = 0.05, respectively). No correlation was observed between the mitochondrial ATP production and the 2-MWT distance. The plasma concentration of oxidized glutathione was negatively correlated with the T_{limQ}, MVCQ values, and the 2-min walk distance values (rho = -0.60, P = 0.03; rho = -0.56, P = 0.04; rho = -0.93, P < 0.0001, respectively). No correlation between the other systemic oxidative stress markers and functional muscle impairment was found. Only TNF-α, among the proinflammatory cytokines, was negatively correlated with both T_{limQ} and MVCQ values (rho = -0.58, P = 0.03; rho = -0.74, P = 0.003; respectively). No association between the TNF-α and the 2-MWT distance was observed. These preliminary results need to be confirmed with larger samples.
disease phenotype and some of these genes might contribute differently to the mitochondrial dysfunction and oxidative stress among different patients or different muscles of a given patient. In a more direct mechanism, several genes involved in susceptibility to oxidative stress are affected by DUX4 expression [24,65]. Furthermore, the acute cell toxicity mediated by DUX4 overexpression in mouse C2C12 myoblasts was alleviated by antioxidants such as ascorbic acid and vitamin E added to their culture medium [24].

We then show that these local alterations are associated with systemic oxidative stress and inflammation. Patients with FSHD have reduced antioxidant capacity and higher oxidative damage than age-matched controls. Some alterations were observed in most patients, such as low levels of zinc and selenium. In addition, patients frequently presented increased levels of ferritin, known to reflect the extent of oxidative stress and inflammation [66]. The significant decrease in ascorbate content suggests that patients with FSHD are subjected to free radical-induced oxidative stress that causes ascorbate consumption in plasma [67,68]. Indeed, ascorbate depletion was associated with a significant increase in lipid peroxidation and oxidized DNA.

Finally, the GSH/GSSG ratio was strongly decreased in FSHD blood samples, as a consequence of GSSG accumulation, indicating a shift to a more oxidized intracellular redox state. The GSH/GSSG ratio decrease might be related to DUX4 activation since the glutathione redox pathway was repressed on DUX4 overexpression in C2C12 cells [24]. Systemic oxidative stress was also accompanied by increased expression of proinflammatory cytokines and chemokines. The mechanisms that trigger the production of inflammatory cytokines and chemokines in FSHD are unknown, but they are likely related to GSSG accumulation [69–71]. Accordingly, TNF-α increase was significantly correlated with the plasma concentration of GSSG (rho=0.62; P=0.02), which is involved in the induction of proinflammatory responses, such as increased TNF-α release [72]. This correlation has important implications for FSHD because it supports the notion of a relationship between oxidative stress and inflammation.

The present data are in agreement with the observation that DUX4 overexpression in mouse or human myoblasts results in repression of glutathione redox pathway components and increased sensitivity to oxidative stress [24]. The correlations between systemic oxidative stress and functional muscle impairment further suggest that muscle weakness could partly result from oxidation of proteins of the contractile apparatus [73–75] and the disruption in mitochondrial energy production. In the long term, high cytokine levels might influence the exercise capacity in patients by affecting muscle function and volume. This is in agreement with our data showing a significant negative relationship between TNF-α and clinical functional assessments.

In conclusion, we show that markers of local and systemic oxidative stress are elevated in FSHD patients relative to controls. This leads us to propose that oxidative stress may contribute to the peripheral skeletal muscle dysfunction in FSHD. During FSHD, sustained ROS generation from inflammatory and mitochondrial origin, coupled with an inadequate antioxidant response, results in inefficient ROS scavenging in muscle and leads to long-term oxidative stress and oxidative damage of the muscle cellular components. If oxidative damage is part of the FSHD pathophysiological mechanism, then treatments that could modulate or delay the onset of the oxidative insult and mitochondrial deficiencies in muscle might be useful for maintaining FSHD muscle functions in the absence of a drug that targets the cause of FSHD.
Acknowledgments

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Appendix A. Technical appendix

Mitochondrial respiration in permeabilized skinned fibers

High-resolution respirometry allowed a routine approach for multiple substrate–inhibitor titrations, to measure the electron transport chain (ETC) activity under physiological conditions. It is an ex vivo approach of the bioenergetic metabolism. Respiratory parameters of the total mitochondrial population were analyzed in situ using saponin-skinned muscle fibers as previously described [55,56]. Respiration rates were recorded in the presence of 10 mM pyruvate and 2 mM malate as respiratory substrates. For each sample, basal oxygen consumption without ADP (state 4; \( V_0 \)) was first recorded and then the ADP-stimulated maximal respiration rate (state 3; \( V_{\text{max}} \)) was determined in the presence of saturating concentrations of ADP (2 mM). The Respiratory Control Index (RCI) was calculated as \( V_{\text{max}}/V_0 \) [76]. At the end of each measurement, cytochrome c was added to evaluate the outer mitochondrial membrane integrity. Fiber bundles were then removed, dried for 48 h, and weighed the next day. Replicates from the same biopsy were used to confirm the precision of the measurements. Respiration rates were expressed in nmol of O$_2$ min$^{-1}$ (mg dry weight)$^{-1}$.

Assessment of the different complexes of the ETC was done by successive respiration measurements after addition to the muscle fibers of 2 mM ADP, 10 mM pyruvate and 2 mM malate, 20 mM rotenone, 10 mM succinate and 2 mM malate, 6.5 mM antimycin, 2 mM ascorbate, and finally 5 mM TMPD. The ADP-stimulated maximal respiration under pyruvate/malate feeding represented the maximal respiration from electron flow through complexes I, III, and IV. The ADP-stimulated maximal respiration under succinate/malate feeding, with rotenone as inhibitor of complex I, represented the maximal respiration from electron flow through complexes II, III, and IV. The ADP-stimulated maximal respiration under TMPD-ascorbate feeding, with antimycin as inhibitor of complex III, represented the maximal respiration from isolated complex IV (cytochrome oxidase complex). Fiber bundles were then removed, dried over 48 h, and weighed the next day. Replicates from the same biopsy were used to confirm the precision of these measurements.

Citrate synthase activity

Muscle extracts were homogenized in 10 mmol/L Tris HCl (pH 7.4). Citrate synthase activity was measured with 0.5 mmol/L 2-nitrobenzoic acid, 100 mmol/L Tris HCl (pH 8.0) and 0.1% (vol/vol) Triton 100-X. Citrate synthase activity was monitored by recording the changes in absorbance at 412 nm at 37 °C for 2.5 min and normalized to tissue weight.

Efficiency of mitochondrial ATP production in permeabilized fibers

Under identical conditions, the rate of mitochondrial synthesis of ATP in skinned fibers was determined using the ATP bioluminescence assay kit HS II from Roche Diagnostics GmbH (Mannheim, Germany) after addition of 2 mM ADP [57]. Basal respiration without ATP synthesis was measured after addition of 70 μM atractyloside and 1 μM oligomycin. Ten aliquots were collected from the oxygraph chamber at various time points after ADP addition, quenched in 100 μl DMSO, and diluted in 5 ml ice-cold distilled water. Standardization was performed with known quantities of ATP measured under the same conditions. ATP synthesis rate was expressed in nanomoles of ATP produced per minute and per milligram of fiber (dry weight). The efficiency of oxidative phosphorylation was taken as the ratio between the ATP synthesis rate and the oxygen consumption rate and was expressed as ATP/O [57].

Measurements of ATPase subunits and cytochrome c in muscle biopsies by Western blotting

Thirty micrograms of each muscle biopsy was lysed in Laemmli buffer as previously described [30]. The following primary antibodies were used: anti-ATP synthase alpha and beta (BD Biosciences) and cytochrome c (Santa Cruz Biotechnology). Anti-alpha sarcomeric actin (Sigma) was used as loading control. Protein levels were quantified using the Image J Launcher Software (NIH).

Transmission electron microscopy

Freshly obtained muscle samples (5 mg) were fixed in 3.5% (vol/vol) glutaraldehyde in 0.1 M phosphate buffer, pH 7.4, at 4 °C overnight. Samples were then prepared as previously described [77]. Electron microscopy was carried out at the Centre Régional d’Imagerie Cellulaire (CRIC) of Montpellier (France).

Evaluation of oxidative stress in FSHD muscle biopsies

Detection by fluorescence microscopy of lipofuscin, a product of the oxidation of unsaturated fatty acids, was based on its autofluorescence. Ten-micrometer quadriceps sections were mounted in Mowiol and then viewed under a fluorescence microscope with an FITC (DM510) filter. Muscle membrane staining was performed using rabbit antibody H4 (1:500) directed against dystrophin as previously described [78]. Immunoreactivity was detected with goat anti rabbit IgG antibodies coupled to Alexa 647 (Invitrogen 1:1000). Data were analyzed with the Histolab software (Microvision 6.1.0; license number 3105). Results were expressed as number of lipofuscin granules per fiber.

The amount of 4-hydroxy-2-nonenal-modified proteins was determined by Western blotting according to the manufacturer’s recommendation (Alpha Diagnostic International; HNE11-S). Muscle thiobarbituric acid-reactive substances, used as a marker of muscle lipid peroxidation, were determined spectrophotometrically using the method described by Ohkawa et al. [79]. The final results were expressed in nM/mg proteins. The TBAR coefficient of variation (to assess reproducibility) was less than 10%. Protein oxidation was measured by evaluating the levels of carbonylated proteins by immunoblotting (S7150, Oxyblot protein oxidation detection kit; Millipore; Molsheim, France). Muscle protein carbonyl content was calculated by adding the integrated density of individual protein bands (Alpha Innotech Corporation, San Leandro, CA) obtained by Western blot analysis [80].

The antioxidant response was analyzed by Western blotting using antibodies anti-catalase 11A1 (LF-MA0010, Euromedex, Souffelweyersheim, France), which catalyzes the breakdown of hydrogen peroxide to oxygen and water, anti-glutathione reductase GR (LF-PA0056, Euromedex, Souffelweyersheim, France), an enzyme that reduces glutathione disulfide (GSSG) to the sulfhydryl form GSH, and anti-manganese superoxide dismutase MnSOD (SOD-110, Euromedex, Souffelweyersheim, France), and
anti-copper–zinc superoxide dismutase Cu-ZnSOD (SOD-101, Euromedex, Souffelweyersheim, France) enzymes, which catalyze the reduction of superoxide anions to hydrogen peroxide. Alpha sarcomeric actin (A2172, Sigma-Aldrich, Saint-Quentin Fallavier, France) was used as loading control. For all primary antibodies, dilution was 1:1000. Secondary antibodies were coupled to horseradish peroxidase (HRP) and were sheep anti-mouse and donkey anti-rabbit IgG antibodies (respectively, NA931 and NA934, GE Healthcare Life Sciences, Velizy-Villacoublay, France) with 1:5000 dilution. Protein staining intensities were quantified using the ImageJ Launcher software (NIH). However, the small biopsy size precluded enzymatic activity assays.

**Evaluation of oxidative stress in FSHD blood**

**Antioxidants**

For vitamin C determination, 0.5 ml plasma was immediately transferred in ice-cold tubes containing 0.5 ml of 10% metaphosphoric acid and then frozen in dry ice. Analysis of vitamin C was performed using a spectrophotometric method and the reduction of 2,6-dichlorophenolindophenol (Perkin Elmer Lambda 40, Norwalk, USA) [81]. Plasma vitamin A, vitamin E (α- and γ-tocopherols), and β-carotene were determined simultaneously by HPLC (Alliance Waters, USA) coupled with a diode array detector [82]. Blood levels of vitamin E were normalized to those of a reference lipid (i.e., cholesterol) [83], which were determined by an enzymatic method with cholesterol oxidase. The ratio of vitamin C/α-tocopherol was used as a potential risk factor for cardiovascular disease [84]. The γ/α-tocopherol ratio was determined as a sensitive index of α-tocopherol ingestion [85]. Total GSH and GSSG were determined in whole blood using the GSH/GSSG-412 kit (Bioxytech, Oxis International Inc., Portland, WA, USA). SOD and GPx enzymatic activities in whole blood were determined with the Ransod and Ransel kits (Randox, England) and expressed as UI/g of hemoglobin. Ubiquinone analysis was performed by HPLC with a diode array detector (PDA 2996, Waters, USA) and the Co-Enzyme Q10 kit (68100) developed by Chromsystems (Germany).

**Trace elements**

Plasma levels of selenium, copper, and zinc were determined by inductively coupled plasma–mass spectroscopy [86].

**Markers of lipid peroxidation and DNA damage.** The analysis of lipid peroxides as markers of oxidative damage of lipids was performed with a commercial kit (Oxystat, Biomedica Gruppe, Austria). Briefly, the peroxide (-OOH) concentration was determined spectrophotometrically by reaction of the biological peroxides with peroxidase and 3,30,50-tetramethylbenzidine as substrate. Oxidized LDL in plasma samples was determined spectrophotometrically with a competitive enzyme-linked immunosorbent assay (ELISA) kit (Immunodiagnostik, Germany). The titer of free antibodies (IgG) against antibodies bound to oxidized LDL (Ab-Ox-LDL) was assessed with a commercial enzyme immunoassay (Biomedica Gruppe, Austria) using Cu2+-oxidized LDL as antigen. 8-Hydroxy-2-deoxyguanosine (8-OHdG), an oxidative DNA damage adduct, was analyzed in fresh urine samples with the new 8-OHdG ELISA kit that uses the anti-8-OHdG monoclonal antibody (clone N45.1) developed by the Japan Institute for the Control of Aging, Nikken SEIL Co., Shizuoka, Japan. 8-OHdG concentrations were standardized to the urine dilution by expressing the data as the 8-OHdG/urinary creatinine ratio.

**Miscellaneous.** Ferritin concentration was determined with the Fe kit from Roche (Roche Diagnostics) (Belgium). Myeloperoxidase, a marker of leukocyte activation, was determined with the Elisa K 6631 kit from Immunodagnostik, Germany.

**Determination of ascorbyl free radical (AFR) content in plasma by electron spin resonance (ESR).** Blood samples from patients with FSHD and healthy controls were collected in heparinized tubes which were immediately inverted five times and left at room temperature for 2 h. Plasma samples were then obtained by centrifugation (4700g: 4 C; 10 min) and immediately frozen in liquid nitrogen. Each sample was rapidly thawed, diluted in 1:1 v/v dimethyl sulfoxide (DMSO), and placed in 50-μl calibrated glass capillary tubes (Hirschmann Lab., Germany) sealed at both ends with Critoseal. Tubes were fitted into the cylindrical cavity of a Bruker ESP 300 ESR spectrometer (Karlsruhe, Germany) operating at X-band (9.87 GHz) with 100 kHz modulation frequency, 10 mW microwave power, 0.787 G modulation amplitude, 2.5×10^5 receiver gain, and 1.92 ms time constant. Spectral recording was initiated at room temperature 75 s after thawing and the relative concentration of the AFR-DMSO ESR doublet (aH=1.83 G), which resulted from the signal averaging 10 scans at 1.07 G/s sweep rate, was calculated by double integration of the simulated signal using the Winsim software [87].

**Blood levels of inflammatory markers**

Serum was collected by centrifugation, aliquoted, stored, and processed at the end of the study by blinded staff. Inflammatory markers were simultaneously identified using the BD Cytometric bead array immunoassay (CBA) (BD Bioscience, San Jose, CA, USA). CBA is a multiplexed bead assay in which a series of spectrally discrete particles can be used to capture soluble analytes. Analytes are then quantified by flow cytometric analysis of their fluorescence-based emission. The method employs a series of different particles that are stably labeled with a discrete level of fluorescent dye whose emission wavelength is read at ~650 nm. The beads within each group are covalently coupled with antibodies that can specifically capture a particular type of molecule present in biological fluids.

**Appendix A. Supplementary material**

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.freeradbiomed.2012.06.041.

**References**
