Morphological abnormalities in mitochondria of the skin of patients with sporadic amyotrophic lateral sclerosis

Alteraciones morfológicas en las mitocondrias en la piel de enfermos con esclerosis lateral amiotrófica esporádica

Gabriel E. Rodríguez[1,2], Deniselle M. Claudia González[3,4], Gisella M. Gargiulo Monachelli[1,4], Juan J. López Costa[5], Alejandro F. de Nicola[3,4], Roberto E. P. Sica[2]

ABSTRACT

Objectives: Mitochondrial dysfunction has been reported in the central nervous system, hepatocytes and peripheral blood lymphocytes from patients with sporadic amyotrophic lateral sclerosis (SALS). However, the status of skin mitochondria has not been reported, in spite of the fact that SALS patients present skin abnormalities. The objective of the present study was to compare mitochondrial ultrastructural parameters in keratinocytes from patients with SALS and healthy controls. **Methods:** Our study was based on the analysis of 112 skin mitochondria from 5 SALS patients and 99 organelles from 4 control subjects by electron microscopy. **Results:** Computerized image analysis showed that mitochondrial major axis length, area and perimeter of the organelle were significantly smaller in SALS respect of healthy control subjects. Morphologically, SALS mitochondria presented cristolysis and breakage of the outer membrane. **Conclusions:** Mitochondrial dysfunction in the skin may possibly reflect changes occurring in mitochondria of the central nervous system. The analysis of mitochondrial morphology in this tissue may be of value to follow disease progression and, eventually, the effectiveness of current therapies for SALS.

Key words: amyotrophic lateral sclerosis, electron microscopy, keratinocytes, mitochondria.

RESUMEN

Objetivos: Existen alteraciones en la función mitocondrial en el sistema nervioso central, en hepatocitos y en linfocitos de sangre periférica en SALS. Aunque, no se ha estudiado si existen cambios estructurales en las mitocondrias de la piel. Nuestro objetivo fue comparar la ultraestructura de mitocondrias en queratinocitos de enfermos con SALS con la de controles sanos. **Método:** Fueron analizadas en el microscopio electrónico 112 mitocondrias dérmicas de 5 pacientes y 99 provenientes de 4 controles. **Resultados:** EL análisis computarizado mostró que el eje mayor mitocondrial, el área y el perímetro de las organelas fueran significativamente menor que en controles. Morfológicamente, las mitocondrias de SALS presentaron cristólisis y ruptura de la membrana externa. **Conclusión:** La alteración mitocondrial en la piel posiblemente refleje cambios que también ocurran en las mitocondrias neuronales. Este análisis morfológico de las mitocondrias podría tener valor en el seguimiento de la enfermedad y eventualmente en la evaluación de la efectividad de futuras terapias.

Palabras-Clave: esclerosis amiotrófica lateral, microscopía electrónica, queratinocitos, mitocondria.

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disease that compromises motor neurons of the spinal cord and brainstem. About 10 to 20% of ALS cases belong to the familial form (FALS); 2 to 5% of them present a mutation in the superoxide dismutase type I (SOD1) gene. The largest majority of patients belong to the sporadic form (SALS) of unknown etiology. Multiple factors have been held responsible for motor neuron degeneration, including glutamate excitotoxicity, increased generation of reactive oxygen species (ROS), abnormal mitochondrial function, altered calcium homeostasis, deposits of cytoplasmic clusters of protein aggregates, proteasomal disability, impaired

[1]Neurology Department, Hospital Ramos Mejía, Buenos Aires, Argentina;

[2]Department of Science and Technology, School of Medicine, University of Buenos Aires (UBA), Buenos Aires, Argentina;

[3] Department of Human Biochemistry, School of Medicine, UBA, Buenos Aires, Argentina,

[4]Laboratory of Neuroendocrine Biochemistry, Institute of Medicine and Experimental Biology – CONICET, Buenos Aires, Argentina;

[5] Institute of Cell Biology and Neuroscience "Prof. E. de Robertis", School of Medicine, UBA, Buenos Aires, Argentina.

Correspondence: Gabriel E. Rodríguez; Neurology Department; Hospital Ramos JM Ramos Mejía; Urquiza 609; CP: 1221 Buenos Aires - Argentina; Email: gerodrig@intramed.net

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axoplasmic transport, changes of cytoskeletal architecture and inflammation[1].

Participation of oxidative stress in SALS is supported by the increased content of oxidative markers in biological fluids[2-4]. Pathological overproduction of ROS could arise from either increased production or decreased activity of antioxidant scavengers. In pathological situations, mitochondria have been implicated in generation of ROS, calcium-mediated excitotoxicity and intrinsic apoptotic pathways[5].

There is substantial evidence for mitochondrial dysfunction in SALS, supported by morphological, molecular and functional observations in human patients, transgenic mice overexpressing mutant SOD1 and the Wobbler mouse mutant. Afifi and colleagues[6] first reported abnormal mitochondrial morphology in the atrophic muscles of SALS patients and, subsequently, mitochondrial abnormalities have been reported in the central nervous system[7-10], liver cells[11] and peripheral blood lymphocytes[12] of SALS patients. Muscle biopsies in SALS have shown increased mitochondrial volume associated to increased calcium levels[13]; also, reduced activity of mitochondrial respiratory chain complex I and complex IV in skeletal muscle and the spinal cord have been reported[14,15]. Along this line, electron microscopy of motor neurons from animal models of ALS, including SOD1 transgenic and Wobbler mice, revealed prominent mitochondrial abnormalities, such as vacuolation, crystolysis, edema and rupture of outer membranes[16-18].

Recent findings have greatly expanded our understanding of the mitochondria role in the pathogenesis of neurodegenerative diseases. Indeed, mitochondrial dysfunction and oxidative stress occur early in all major neurodegenerative diseases, and there is strong evidence that mitochondrial impairment aggravates disease outcome[19].

In spite of the fact that SALS has been considered a disease of motor neurons, it might also compromise other organs, such as the skin. In this respect, SALS patients are resistant to the development of pressure sores (decubitus ulcers). The skin of these patients feels supple, like tanned leather, and loses elasticity. Fullmer and colleagues[20] have found extensive alterations within the connective tissues of the dermis in a high percentage of SALS patients. The reported skin changes include elastosis, increased mucopolysaccharides, local areas of regenerated connective tissue and altered collagen[21,22]. Deposits of amorphous material consisting of hyaluronic acid have been found in skin biopsies from SALS patients, but not from controls and, interestingly, the amount of this mucopolysaccharide acid correlates with disease length[23-25]. Therefore, it is likely that mitochondrial abnormalities are not restricted to the central nervous system (CNS) and may appear in tissues yet unexplored. Taking into consideration this possibility, the objective of our study was to compare mitochondrial ultrastructural parameters in keratinocytes from the skin of SALS patients and healthy controls.

METHODS

We analyzed 112 mitochondria from 5 patients with the diagnosis of definite SALS, based on El Escorial (Arlie House modified) criteria[26,27] and 99 from 4 healthy controls without any signs of neurological disease. We performed a skin biopsy with a punch device number 3 after local anesthesia with 3% lidocaine. Keratinocytes measured in the study were those near the basement membrane. The latter was identified morphologically by electron microscopy. The research protocol was approved by the Ethics Committee of the Ramos Mejía Hospital and informed consent was obtained in writing from each individual tested.

Electron microscopy

The biopsies were taken from the skin of the shoulder and small blocks of tissue were obtained by cutting longitudinal sections of 3–5 mm maximum thickness. Blocks were immersed immediately for 2 h in phosphate buffered 2.5% glutaraldehyde. After overnight washing in 0.1 M sodium phosphate buffer, tissue blocks containing the epidermal region were postfixed in 1% OsO_4 in 0.1 M phosphate buffer pH 7.4 for 1 h and stained with 1% uranyl acetate. Afterwards, tissue blocks were dehydrated and flat-embedded in Durcupan (Fluka Chemic AG, Sweden). Semithin sections (0.8–1μm) were stained with toluidine blue for light microscopy (LM) observations. For electron microscopy (EM), ultrathin sections (60–70 nm) were obtained with a Reichert ultramicrotome (Vienna, Austria) from keratinocytes. Sections were stained with lead citrate, examined at 20000 and 40000 X magnifications and photographed using a Zeiss 109 electron microscope.

Morphological parameters of skin mitochondria were analyzed using a computerized image analyzer (OPTIMAS 6.02)[18]. Thus, quantitative analysis comprised determination of mitochondrial axis length, area, perimeter, size and the presence or absence of crystolysis, edema and membrane integrity.

Statistical analysis

Data are expressed as means ±2 SEM. Comparisons between groups were calculated by Student´s *t*-test for independent samples, and $p<0.05$ was considered statistically significant. All statistics were carried out using SPSS software version 15.0 (SPSS, Inc. Chicago).

RESULTS

Clinical data of the studied population are shown in Table. The average age in ALS group was 43.8 (±11.71) and 46.25 (±13.25) years in the control group (p:ns). Observations of mitochondrial morphology in keratynocytes from SALS

patients revealed important differences with healthy controls. Thus, the mitochondrial major axis length was significantly lower in SALS patients in comparison to the organelles from control keratynocytes (SALS: 0.358±0.150 μm; controls: 0.440±0.166 μm; mean±2 SEM; p<0.001) (Fig 1A). Fig 1B shows the comparison of mitochondrial area in the two groups obtained by image analysis. Mitochondrial area was also significantly reduced in SALS *vs.* control mitochondria (SALS: 0.076±0.061 μm^2; controls 0.105±0.072 μm^2; p<0.001). The same trend was found for the mitochondrial perimeter, which was decreased in SALS keratynocytes (1.106±0.445 μm) compared with controls (1.349±0.481 μm; p<0.0001) (Fig 1C).

Typical microphotographs obtained by electron microscopy of keratynocyte ultrastructure are shown in Fig 2. Upper and lower microphotographs on the left hand side confirmed the decreased size of SALS mitochondria (asterisk, Fig 2A, C) as opposed to the organelles of control keratynocytes (Fig 2B, D). Whereas most mitochondria from controls showed the expected size, occasionally some smaller mitochondria were observed as well (Fig 2B; arrowhead).

Higher magnification photomicrographs (40000 X) allowed a further comparison of intramitochondrial ultrastructure (Fig 3). In this case, several mitochondria from SALS patients showed increased signs of crystolysis, which were totally absent in controls (arrow, Fig 3B). In addition, 4.46% of the total number of mitochondria obtained from the skin of SALS patients presented a disorganized ultrastructure, including disrupted cristae and loss of membrane integrity near the poles (Fig 4A, arrowhead). This abnormality was not observed in healthy controls (Fig 4B). In summary, electron microscopy observations of SALS skin mitochondria revealed that severe pathological changes, including decreased size, edematous matrix and damage of mitochondrial membranes, were specifically associated with the neurodegenerative condition.

DISCUSSION

The present investigation demonstrated pronounced morphological abnormalities in skin keratinocytes of SALS patients. Low power microscopy of skin biopsies in our series of patients showed mitochondria of small size, along with reductions in area and perimeter. In addition, high power microscopy evidenced damage to cristae, edema and altered membrane continuity. These findings are in consonance with mitochondrial abnormalities reported in the central nervous system of SALS patients and animal models of motor neuron degeneration[8,10]. Our present investigation suggests that changes taking place in skin mitochondria may reflect to some extent the mitochondrial abnormalities of the degenerating nervous system.

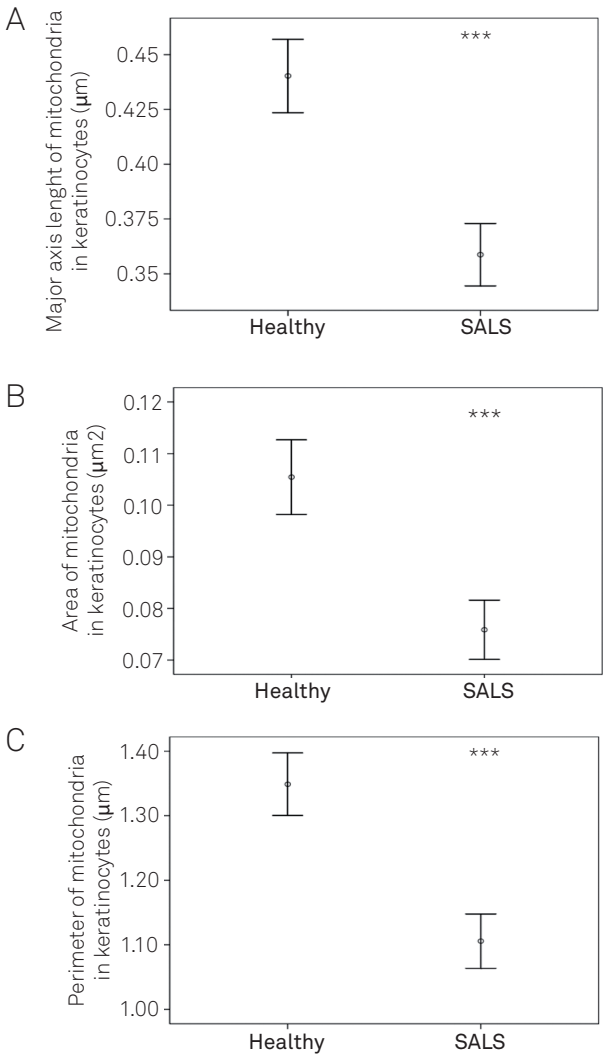

Fig 1. (A) Quantitation of the mitochondrial major axis length (mean±2 SEM) in keratinocytes from healthy subjects (Healthy) and sporadic amyotrophic lateral sclerosis (SALS) patients. Statistical analysis demonstrated significant lower values in the mitochondrial major axis length in keratinocytes from SALS patients (***p<0.001 *vs.* healthy controls). (B) Computarized analysis of the mitochondrial area (mean±2 SEM) in keratinocytes from healthy subjects (Healthy) and SALS patients. A significant decrease in this parameter was demonstrated in keratinocytes from SALS patients (***p<0.001 *vs.* healthy controls). (C) Computerized image analysis of the mitochondrial perimeter. Mitochondrial perimeter was significantly decreased (SALS) respect of healthy subjects (***p<0.0001).

Table. Demographic and clinical characteristics of sporadic amyotrophic lateral sclerosis patients and controls.

ALS Patients	Sex	Age	ALS type	Time onset to diagnosis (months)
1	Male	41	Spinal	14
2	Male	46	Spinal	7
3	Male	26	Bulbar	48
4	Fem.	58	Spinal	7
5	Male	48	Spinal	35
Controls				
6	Male	37		
7	Fem.	38		
8	Male	45		
9	Fem.	65		

ALS: amyotrophic lateral sclerosis.

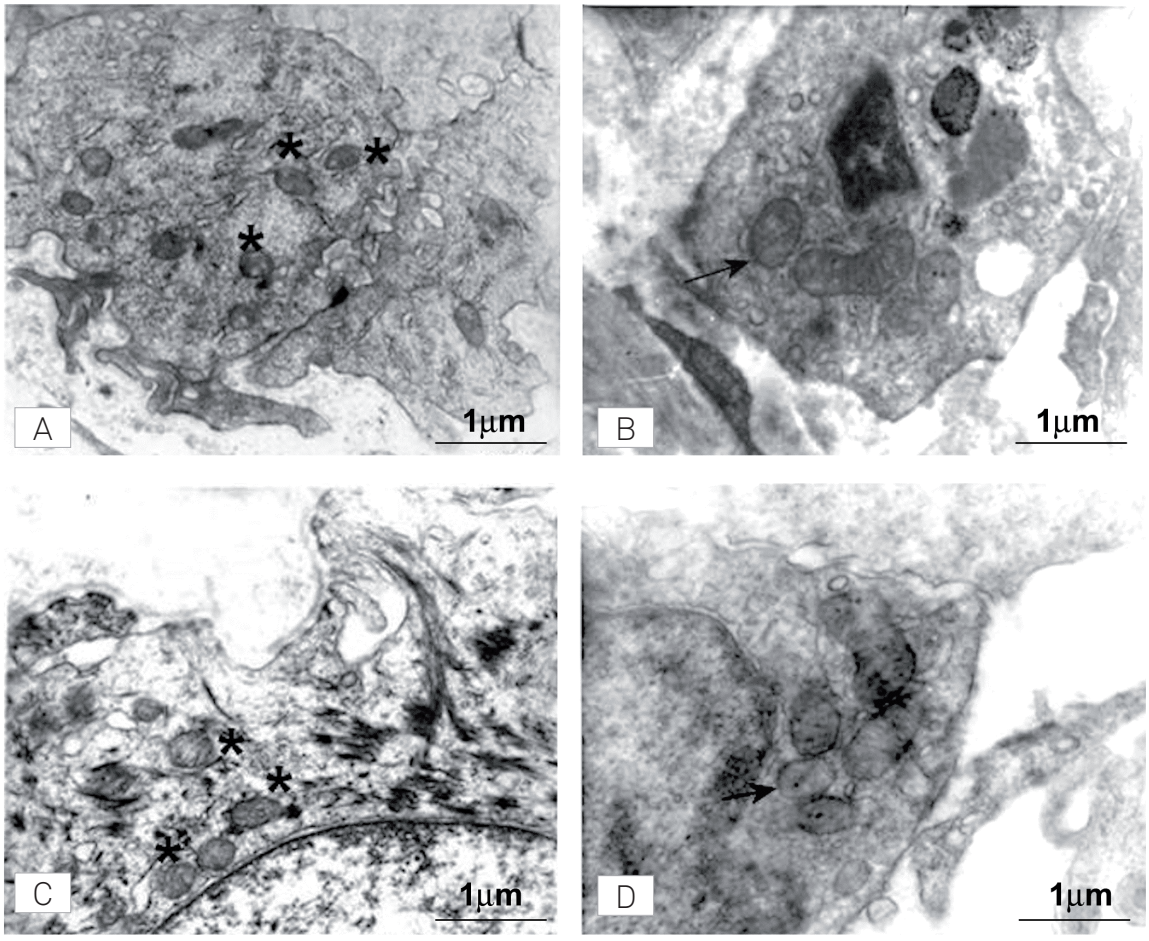

Fig 2. Electron microscope microphotographs in keratinocytes from control subjects and sporadic amyotrophic lateral sclerosis (SALS) patients. (A and C) SALS keratinocyte showing mitochondria of lower size (asterisk) than those found in healthy controls (B and D). Magnification: 20000 X.

Abnormal mitochondria is an important source of ROS, but also become damaged when handling of ROS is unbalanced[5]. The involvement of increased oxidative stress involving mitochondria is a likely explanation, albeit probable incomplete, of SALS neurodegeneration. In this respect, increased mitochondrial volume in motor nerve terminals of SALS patients was first described by Siklós et al in 1996[13]. Other reports also found morphologic abnormalities of mitochondria in SALS patients, either in liver biopsy specimens[11] or in axons, which show accumulation of mitochondria as well[13]. Interestingly, conglomerates of dark mitochondria at the presynaptic terminals of anterior spinal motor neurons were described by Sasaki and colleagues[28], suggesting mitochondrial involvement in disabled synaptic contacts between lower motor neurons and their inputs. In addition, functional abnormalities of the mitochondria were described in the CNS, liver and muscle of SALS patients[10,11,14]. These changes suggest that the neurochemical basis for the observed morphological changes are widespread among different tissues and may consist in impaired mitochondrial bioenergetics, loss of membrane potential, reduced calcium buffering capacity and disrupted calcium homeostasis[29].

In our investigations, we resorted to explore skin mitochondria, because the skin may be, as well, a target for the SALS disease´s putative causes. Clinical signs of skin pathology described in the Introduction are accompanied by changes of the transactivation-responsive DNA-binding protein-43 (TDP-43), one of the major component of the ubiquitin-positive inclusions in SALS[30]. However, to date, no abnormalities had been described in the mitochondria of keratinocytes of these patients. Considering that mitochondria of liver, muscle and other tissues are affected, it became rational to assume that changes would also be seen in the mitochondria of keratinocytes, reinforcing the concept advanced by Siklós et al.[13], that changes of mitochondrial area might be a marker of dysfunction.

Although mitochondrial function was not evaluated in our study, rupture of the mitochondrial membranes was one of the findings in the skin of SALS patients. It is known that the intrinsic apoptotic pathway originating in mitochondria is activated by ROS improperly handled by the cell´s anti-

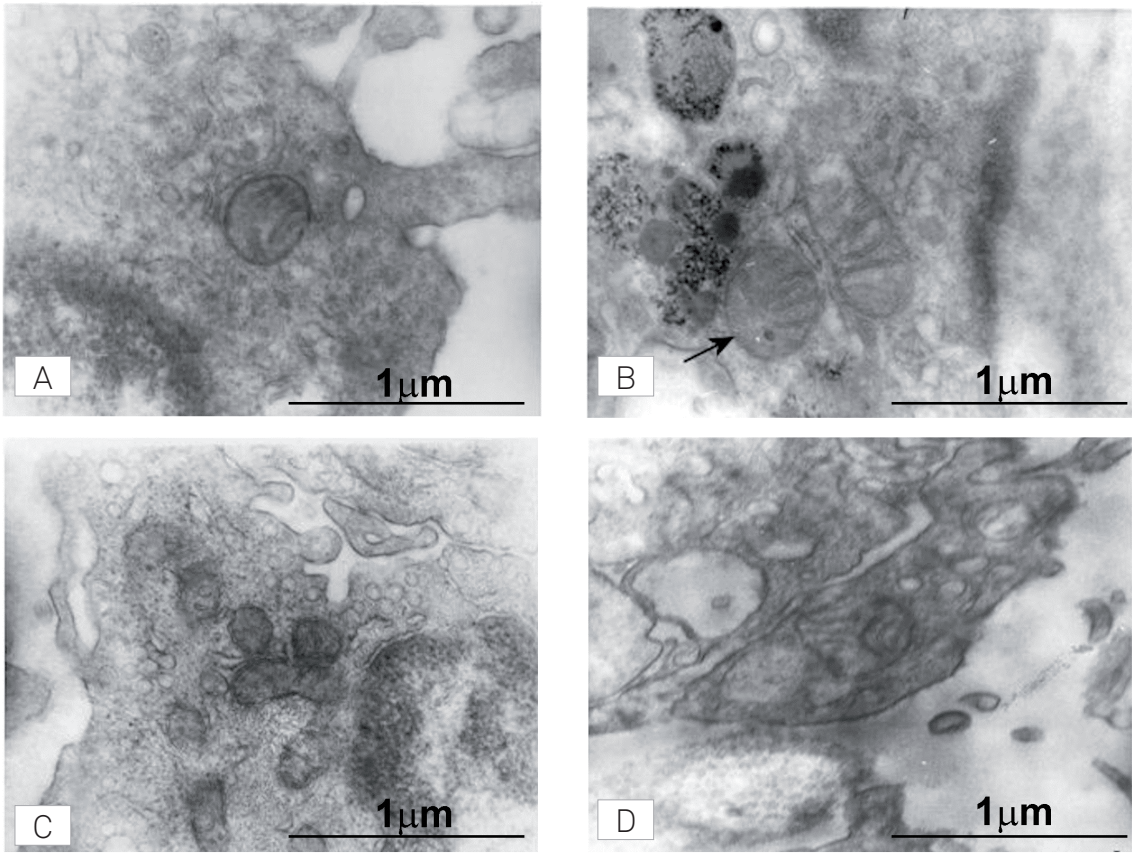

Fig 3. High-power electron microscopy photomicrographs showing smaller mitochondria in sporadic amyotrophic lateral sclerosis (A, C) than in healthy controls (B, D). Magnification: 40000 X.

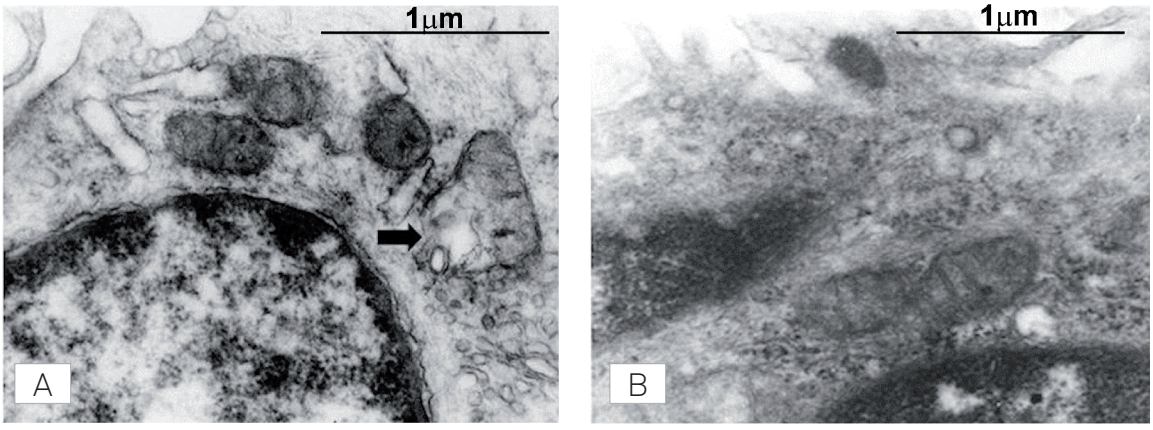

Fig 4. Electron microscopy microphotographs from keratinocytes obtained from sporadic amyotrophic lateral sclerosis (SALS) and healthy subjects. The arrow marks an abnormal mitochondrion in a keratinocyte obtained from a SALS patient, showing cristolysis and complete rupture of the outer membrane (A). Mitochondria from control subject (B). Magnification: 40000 X.

oxidant enzymatic machinery[31]. One of the typical morphological aspects of apoptosis is the breakdown of the external membrane, which causes the release of cytochrome *c*. The latter combines with the APAF molecule and caspase-9 to form the apoptosome, leading to programmed cell death[32].

From this point of view, the morphological changes of skin mitochondria demonstrated in this investigation may be the systemic expression of mitochondrial malfunction yielded by excessive ROS. It is presently unknown in tissues different from the CNS if local factors are responsible for higher levels of ROS or if they depend on an indiscriminate general state of oxidative stress[33]. Thus, there might be a systemic imbalance of ROS associated to this neurodegenerative disease that could explain ultrastructural lesions of skin mitochondria. In

relation to this subject, increased serum levels of thiobarbituric acid reactive substances (TBARS) in SALS patients have been preliminary demonstrated in our laboratory (unpublished results).

In the present study, morphological changes found in SALS skin were restricted to mitochondrial size, area and membrane integrity. The altered parameters could result from the action of an environmental stressor whose intensity might produce a swift cell division in association to the breakdown of the mitochondrial membrane.

Thus, we suggest that mitochondrial damage may be a more widespread phenomenon than previously thought in SALS, despite the fact that the CNS seems to be the main structure that clinically express the various semiological features characterizing the illness.

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