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Bioimpedance spectroscopy characterization of Myalgic Encephalomyelitis/ Chronic Fatigue Syndrome (ME/CFS) peripheral blood mononuclear cells

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Abstract

Myalgic Encephalomyelitis/Chronic Fatigue Syndrome (ME/CFS) is a disabling and chronic disease, importantly related to the current COVID-19 pandemic. Currently, there are no specific laboratory tests to directly diagnose ME/CFS. In this work, the use of impedance spectroscopy is studied as a potential technique for the diagnosis of ME/CFS. A specific device for the electrical characterization of peripheral blood mononuclear cells was designed and implemented. Impedance spectroscopy measurements in the range from 1 Hz to 500 MHz were carried out after the osmotic stress of the samples with sodium chloride solution at 1M concentration. The evolution in time after the osmotic stress at two specific frequencies (1.36 kHz and 154 kHz) was analyzed. The device showed its sensitivity to the presence of cells and the evolution of the osmotic processes. Higher values of impedance (around 15% for both the real and imaginary part) were measured at 1.36 kHz in ME/CFS patients compared to control samples. No significant difference was found between patient samples and control samples at 154 kHz. Results help to further understand the diagnosis of ME/CFS patients and the relation of their blood samples with bioimpedance measurements.

Introduction

Myalgic Encephalomyelitis / Chronic Fatigue Syndrome (ME/CFS) is a multi-system and complex disease. Due to its heterogeneous expression and the absence of internationally agreed diagnostic criteria (there are currently several, such as FUKUDA [1], IOM [2] or Canadian Consensus [3], among others), it is difficult to know its prevalence and real incidence. ME/CFS is a disease that affects people of all ages, being more frequent in women than in men, presenting a 1:4 ratio, with a peak of appearance of the disease at 10-19 years and at 30 -39 years. However, it can occur at any age, and usually has a mean diagnosis time of 5 years, involving visits to multiple specialists [4]. It is difficult to determine the prevalence of ME/CFS, although it is estimated to be between 0.4% and 2.5% of the global general population, and it is currently growing, according to recent references [5, 6]. Researchers have not yet found the causes of ME/CFS, even though an infectious episode near the onset of ME/CFS is recounted by 80% or more of patients [7]. There are no specific laboratory tests to diagnose ME/CFS directly. Therefore, it is of utmost importance to research into new techniques for the correct diagnosis of ME/CFS. COVID-19 pandemic has also stressed the importance of obtaining these diagnostic tools, considering that many symptoms of ME/CFS are shared with lingering COVID patient symptoms [8].

Bioimpedance has initially been explored as a possible marker for this disease [9]. In that work, the impedance pattern of the ME/CFS samples after the introduction of the hyperosmotic stressor were observed as a unique characteristic of the ME/CFS samples, being different from the response observed among the control samples. In another recent work, based on these initial experiments, impedance spectroscopy was used to study single muscle cells and differentiate normal and oxidatively-stressed cell populations [10]. Oxidative stress was induced to cultured rat L6 skeletal muscle cells, obtaining different dielectric properties of cytoplasm permittivity and conductivity from normally cultured cells.

Although these two works [9, 10] have provided initial insights on the use of impedance-based biomarkers for the disease, with a high potential to be used as a clinical diagnostic tool, the exact mechanisms that influence the bioimpedance measurement and its differentiation between patients and control samples remain elusive. It is necessary to perform more experiments to understand the physical behaviour of these samples and provide further experimental setups for the study of different ME/CFS samples. Different frequencies must be used, in order to clarify the role of intra and intercellular ions, and provide further insight on oxidative processes.

In our study, bioimpedance spectroscopy was used to analyze ME/CFS sample data and compare it with control samples. A specific device for the sensing of samples was implemented. Impedance measurements were carried out in the range from 1 Hz to 500 MHz, enlarging the frequency range used in other studies. We measured the basal impedance of the peripheral blood mononuclear cells (PBMCs) in plasm and later we performed an osmotic stress with sodium chloride solution. We carried out the measurements throughout the process at different impedance frequencies to see if there are differences between patients with ME/CFS and healthy controls after the stress, with the final aim to analyse the viability of using impedance spectroscopy for the diagnosis of ME/CFS, together with the optimal frequencies to be used.

Materials and Methods

Sample preparation and protocol

Four chronic fatigue patients diagnosed according to the Centers for Disease Control (CDC) criteria (three women and one man, with an age range between 31 and 47 years) and four age and gender-matched healthy controls were included in the pilot clinical study, following the requirements of the Ethical Committee of Hospital Universitario Virgen del Rocío, Servicio Andaluz de Salud, Sevilla, Spain [11]. The participants signed the corresponding informed consent.

For each participant, the blood extraction was conducted obtaining one EDTA blood tube and four lithium heparin blood tubes. The lithium heparin blood tubes were centrifuged at 1500 rpm at room temperature for 5 minutes to obtain the plasm. The supernatant obtained after the centrifugation (plasm) was centrifuged again at 1800 rpm at room temperature for 5 minutes to ensure that all the blood cells had been eliminated. The supernatant obtained (plasm) was placed in a new sterile tube.

To obtain the peripheral blood mononuclear cells, a density gradient was created using Ficoll®, following the manufacturer's protocol and the method proposed by [12]. 3 mL of Ficoll® was added in a new sterile conic tube of 15 mL. A dilution of the blood 1:3 with

PBS was made in another sterile conic tube of 15 mL. The dilution was placed carefully over the Ficoll®, with a Pasteur pipette, and was let slid down the tube. That blood dilution plus Ficoll® mix was centrifuged at 800g at room temperature for 30 minutes, obtaining the different phases. The mononuclear cell phase was taken and placed in a new sterile conic tube. Between 8-10 mL of PBS were added and the tube was centrifuged at 250 g at room temperature for 5 minutes. The supernatant was eliminated by turning down the tube, and the last step was repeated to wash the cells. Once we had the cellular pellet, it was resuspended in 1 mL of PBS and a cell count was carried out in a Neubauer Improved camera. The concentration of cells was adjusted to 200,000/mL in 2 mL of the plasm previously obtained.
The cell plus plasm mix was placed in a Petri plate with the implemented electrodes described in the next section, and the bioimpedance was measured with the MFIA instrument, from Zurich Instruments [13], 20 minutes to obtain the basal data. After those 20 minutes, 120 uL of NaCl 1M was added to the mix to stress the cells and the bioimpedance was measured 90 additional minutes.

Bioimpedance measurement setup

The MFIA instrument from Zurich Instruments [13] was used to perform impedance measurements in the range from 1 Hz to 500 MHz. Electrodes for impedance measurements were printed on a glass substrate and covered with gold (ENIG technique) by the company FX PCB [14, 15]. Two interdigitated electrodes with a width of 0.2mm (**Fig. 1A**) were printed and placed in a Petri dish, as shown in **Fig. 1B**. These electrodes were connected to the MFIA instrument with the corresponding 2 electrodes configuration (**Fig. 1C**).

Experiments performed

Initial experiments were carried out to study the impedance and phase of plasm, compare with previous existing results [9] and confirm the sensitivity of the device to the presence of cells. Also, initial experiments were carried out to analyze the difference in the absolute value and phase of the measured impedance after the introduction of the NaCl.
Samples from four chronic fatigue patients and four healthy controls were included in our pilot study, following the protocol explained in the previous section. Real part and imaginary part of impedance were then more thoroughly analysed for the whole duration of the experiment at two specific frequencies (1.36 kHz and 154 kHz) to see any possible differentiation between patient samples and control samples. The first frequency, 1.36 kHz, is located in the alpha dispersion region, as defined in [16] and is related to the effect of extra-cellular ions. The second frequency, 154 kHz, is located in the beta dispersion region, and it is associated with the polarization of cellular membranes and the effect of intra-cellular ions [16]. For both frequencies, the real part and imaginary part of the impedance were compared between patient samples and control samples, at specific intervals (every 30 minutes) throughout the duration of the experiment.

Results and Discussion

As previously commented, our objectives were to study initially the sensitivity of the implemented device to the presence of cells, and the study of the evolution in time of bioimpedance measurements at two specific frequencies, 1,36 kHz and 154 kHz, to further understand the relation of ME/CFS blood samples with bioimpedance measurements at different frequencies from other studies, analysing the possibility of using this technique for the diagnosis of ME/CFS patients with our newly implemented electrodes.

Effect of cells and NaCl stress on impedance measurements

Fig. 2 shows the typical graph obtained for the absolute value and phase of the impedance measured for plasm (without cells) versus frequency. The absolute value of the measured impedance decreases with increasing frequency, from approximately 30 kOhms to values under 10 Ohms, in a similar way as other impedance spectroscopy works with cell suspensions [17], corresponding to a transition from a capacitive to resistive behavior. The phase plot shows that the phase angle of plasm without cells moves towards zero degrees with increasing frequency.
Real part and imaginary part of impedance were more thoroughly analysed for the whole duration of the experiment at two specific frequencies (1.36 kHz and 154 kHz) to see any possible differentiation between patient samples and control samples.
The measurements of the absolute values and phases of the impedance of the samples are similar to the one reported for PBS media without cells reported in [17].

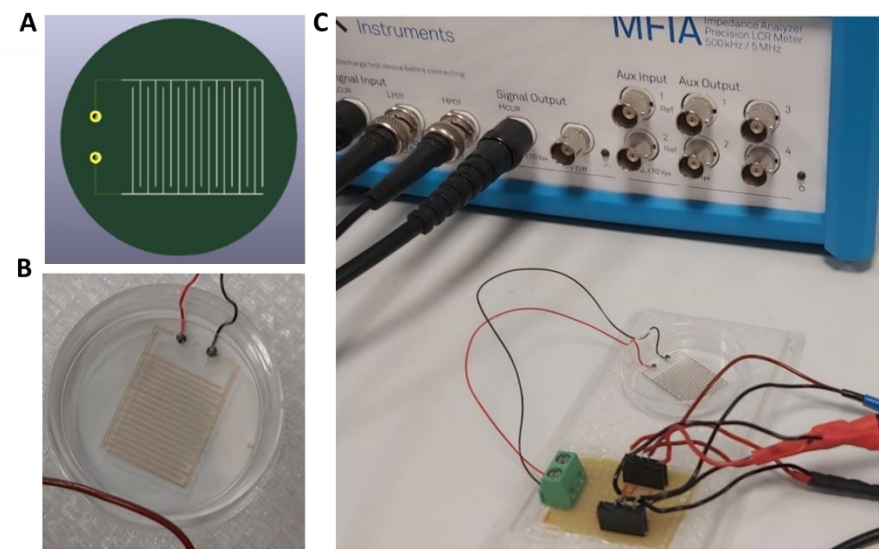

Fig. 1: Bioimpedance measurement setup. A) Interdigitated electrodes PCB design, from [15] **B)** Fabricated electrode used in the Petri dish. **C)** Electrode and terminals connection and complete measurement setup.

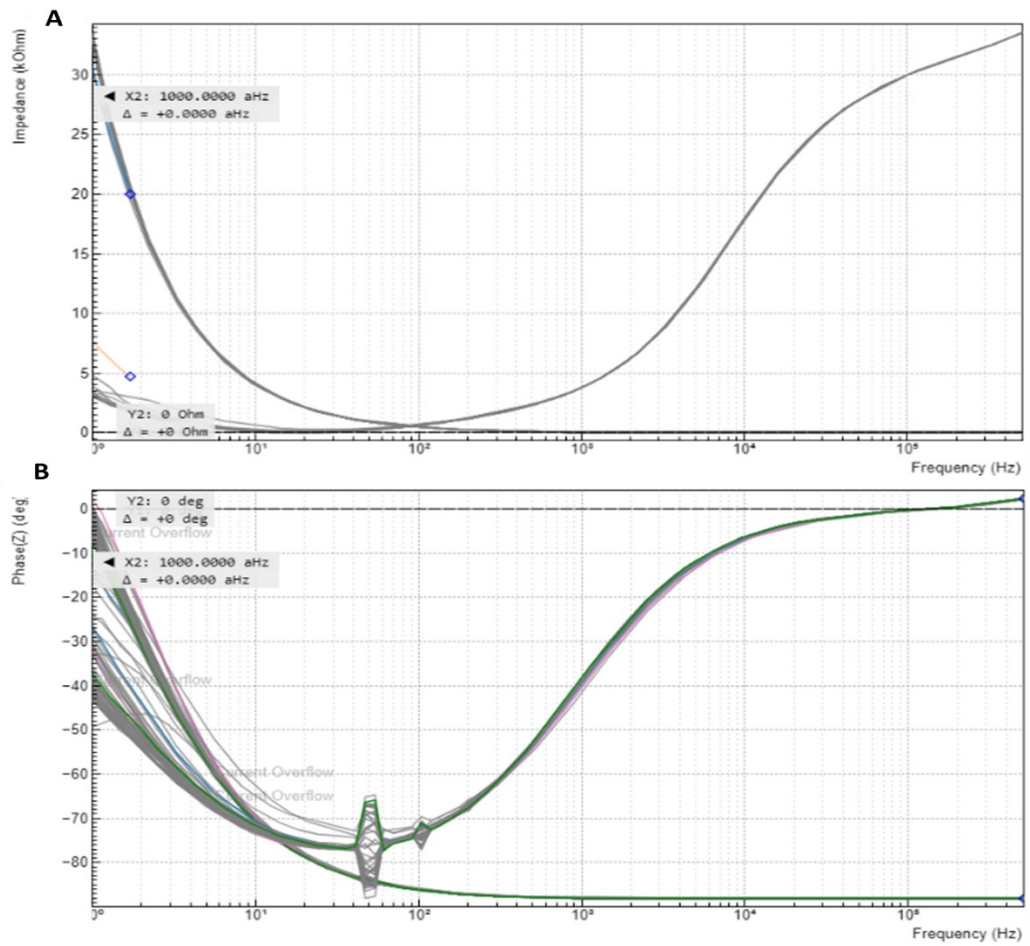

Fig. 2: Impedance analysis of plasm. A) Impedance absolute value (kOhm) versus frequency of the plasm sample from a healthy individual. We can observe the ordinate axis the units for the impedance absolute value. **B)** Impedance phase (deg) of the plasm sample from a healthy individual versus frequency. We can observe the ordinate axis the units for the phase. We also observe electrical noise in the phase at 50 Hz, irrelevant for the study of the samples.

Fig. 3 shows the typical graph obtained for plasm and cells, where NaCl was added to the mix to stress the cells, as described in the Materials and Methods section. It can be distinguished the different behaviour measured once the plasm basal data is obtained (marked in blue, after 20 min of the start of the experiment), the basal data of plasm and cells (marked in red, after 20 min of the insertion of cells), the first measurement after the addition of NaCl (marked in orange), and the final measurement (marked in pink). Significant differences can be observed in the phase of the samples, in the range from 10^3 Hz to 10^5 Hz. An increase in the phase is observed after the insertion of cells, in accordance with previous reported results [17]. Also, an important decrease in the phase is observed after the insertion of NaCl, as could be expected, due to the general increase of conductivity.

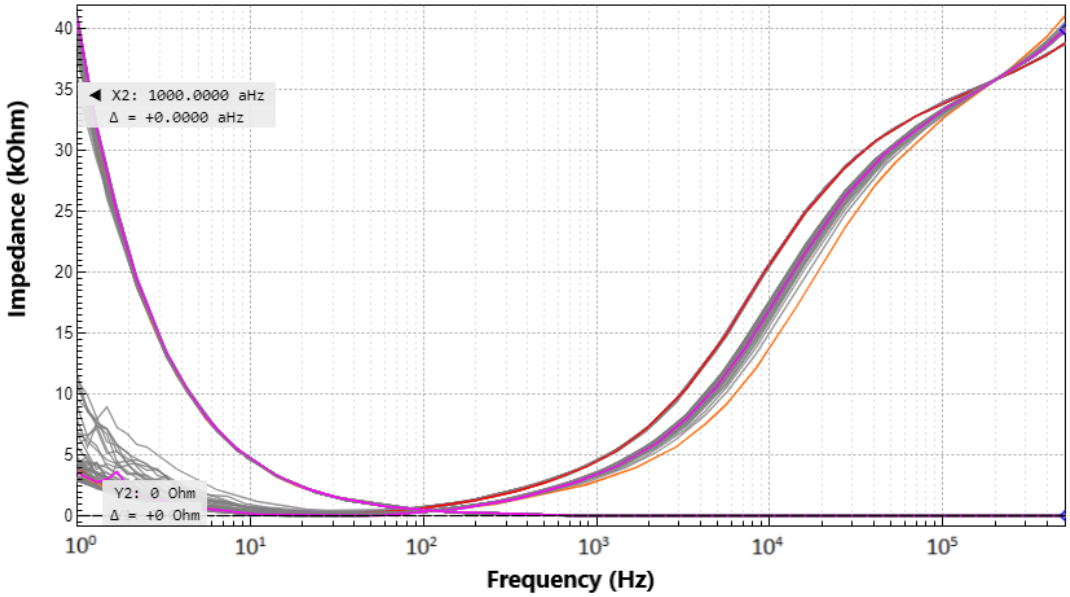

Fig. 3: Impedance analysis of blood samples with cells and NaCL stress. Plasm basal data (blue line, obtained after 20 min of the start of the experiment). Basal data of plasm and cells (red line, after 20 min of the insertion of cells). First measurement after the addition of NaCl (orange line). Final measurement (pink line).

Analysis of impedance measurements at 1.36 kHz

Fig. 4 shows the results of the measured impedance at 1.36 kHz for the different patient and control samples, at different times of the experiments described in the Materials and Methods section. We can observe for all frequencies (**Fig. 4A**) a decrease in the real part of the measured impedance at 30 minutes, as expected after the addition of NaCl solution. The bioimpedance values then start to rise, and stabilize at the end of the experiment. In opposition with [9], no significant increase in the impedance measurements have been found for the patient samples after one hour of measurements, at 1.36 kHz. In [9], electrochemical impedance spectroscopy was carried out at 15 kHz. After a waiting time of 20 minutes to reach a baseline value, a small volume (6 µl) of hyperosmotic stressor was introduced to the cells, obtaining after a transient time higher impedance values in the patient samples in relation with control ones. This different impedance pattern could be due to a possible alteration in the production of ATP as a consequence of deficient mitochondrial metabolism, according to the published work [9].

Fig. 4C and **4D** show the mean values of the real part and imaginary part of the measured bioimpedance at 1.36 kHz. Higher values of impedance (both in the real part and the imaginary part, in absolute values) are measured in ME/CFS patients, in comparison with control individuals, as can be seen in **Fig. 4C** and **4D**, throughout the whole duration of the experiment.

Analysis of impedance measurements at 154 kHz

Fig. 5 shows the results of the measured impedance at 154 kHz for the different patient and control samples. At this frequency, the measured real part values of impedance are lower to the other frequency studied, but the general behaviour in time is similar in both cases.

The real part of the measured impedance decreases at 30 minutes for all cases, as expected after the addition

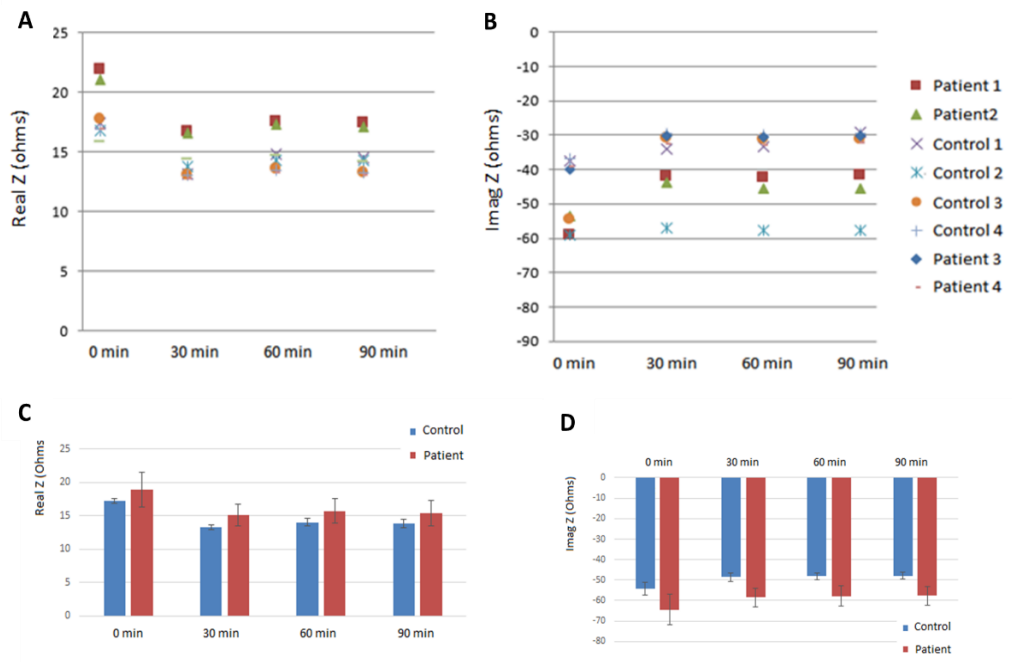

Fig. 4: Real part and imaginary part of bioimpedance at 1.36 kHz. A) Real part of the impedance (Z) for the different samples. The graph shows the evolution in time for the different samples, for the 90 min of duration of the experiment. **B)** Imaginary part of Z for the different samples, at different times of the experiment **C)** Mean value and standard deviation of the real part of Z, at different monitoring times. **D)** Mean value and standard deviation of the imaginary part of Z, at different monitoring times. We can observe higher values of impedance (around 15% both in the real and imaginary part) for ME/CFS patient samples, in comparison with control samples, at the different times of the experiment.

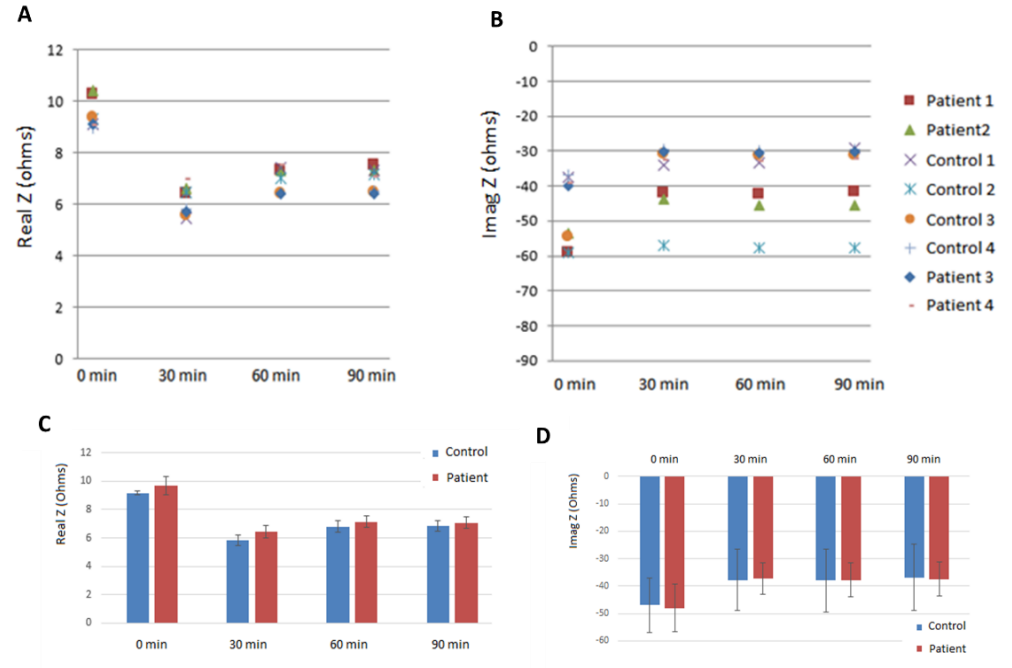

Fig. 5: **Real part and imaginary part of bioimpedance at 154 kHz. A)** Real part of the impedance (Z) for the different samples. The graph shows the evolution in time for the different samples, for the 90 min of duration of the experiment. **B)** Imaginary part of Z for the different samples, at different times of the experiment. **C)** Mean value and standard deviation of the real part of Z, at different monitoring times. **D)** Mean value and standard deviation of the imaginary part of Z, at different monitoring times. At this frequency no significant difference has been found between patient samples and control samples.

of NaCl solution, and then starts to rise, stabilizing at the end of the experiment. In opposition with [9], no increase in the impedance measurements have been found for the patient samples, after one hour of measurements. At this frequency no significant difference has been found between patient samples and control samples. These results suggest that 1.36 kHz may be a better region to study differences between patient and control samples and provide a diagnostic device. This frequency, 1.36 kHz, is located in the alpha dispersion region, suggesting the difference of impedance may be related to the effect of extra-cellular ions.

As previously described, 154 kHz is a frequency located in the beta dispersion region, associated with the polarization of cellular membranes and the effect of intra-cellular ions. Our results differ to the ones presented in [10], where specific intracellular property changes were reported in cultured rat L6 skeletal muscle cells, in the range of frequencies from 9 kHz to 9 GHz, associated with reactive oxygen species. An increase of cytoplasmic conductivity and permittivity was observed in stressed cells, independent of the cell position between the electrodes or their size, in the range of frequencies studied. According to this work [10], the conductivity increase indicates an increase of ions within the cells, consistent with the release of calcium from the endoplasmic reticulum into the cytoplasm to cope with the oxidative stress. A wide variation of the spectra from treated cells was found, likely due to the multiple mechanisms and stages by which reactive oxygen species change the properties of the cytoplasm.

Future experiments

It will be necessary to perform more experiments with our device with both blood samples and cell cultures, to provide further insight on the effect of intra-cellular and extra-cellular ions in the bioimpedance signatures, and see if the difference found at 1,36 kHz is maintained. We are currently working on increasing the sample size to see if this difference in impedance measurement is maintained. Likewise, we want to see if the impedance could be modified if we study PBMCs in their own plasma/serum or if it is modified by adding plasma or serum from healthy subjects and vice versa. In other words, we will study PBMCs from healthy subjects with plasma/serum from patients with ME/CFS to determine if there are extracellular component-factors capable of interacting in the production of cellular energy. If so, we would have to investigate the plasma components (e.g., proteins, exosomes, and lipids) and individual cell types (e.g., T cells) separately.

Similar impedance sensing devices as the one presented in this article have been used to study impedance spectroscopy in cell cultures, specifically in skeletal myoblasts cell cultures [18], so future experiments with cell cultures from ME/CFS patients can be carried out with our device to further study the differences with [10], with the final objective to build a clinically robust diagnostic method for ME/CFS patients. In a second phase, we want to reproduce the study carried out by Ferguson et al [10], measuring the impedance of muscle cells obtained from standard cell cultures as well as from muscle biopsies of patients with CFS/ME, with similar methods as the ones presented in our previous studies with cell cultures [18] and our new measurement setup.

Conclusions

In this work we study the use of impedance spectroscopy as a potential technique for the diagnosis of Myalgic Encephalomyelitis / Chronic Fatigue Syndrome (ME/ CFS). A specific device for impedance measurement was designed and implemented, consisting on two interdigitated electrodes placed on a Petri dish, connected to an impedance analyser, obtaining measurements in the range from 1 Hz to 500 MHz. Peripheral blood mononuclear cells from four chronic fatigue patients and four healthy controls were analysed, after an osmotic stress of the samples with NaCl solution 1M. The real part and imaginary part of impedance were measured for the whole duration of the experiment at two specific frequencies. At 1.36 kHz, higher values of impedance (around 15% both in the real part and the imaginary part, in absolute values) were measured in ME/CFS patients, in comparison with control individuals. At 154 kHz, no significant difference has been found between patient samples and control samples.

Further experiments need to be carried out in order to fully understand bioimpedance measurements and its relation with ME/CFS samples, confirm our results and gain insight into the possible stratification of ME/CFS patients in relation with the proposed diagnostic method.

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Conflict of interest

The authors declare no conflict of interest.

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