ORIGINAL ARTICLE

Revealing alteration of membrane structures during ischema using impedance spectroscopy

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Abstract

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Alterations of membrane structure and function are essential characteristics of cells undergoing ischemia. Noninvasive monitoring of tissue alterations during ischemia and the estimation of the reversibility domain (corresponding to organ capability to fully recover its functions after shifting back to normal blood perfusion) are important for biomedical applications allowing better time management during surgical interventions, especially in organ transplantation.

Due to it's capability to reveal inhomogeneities, as well as it's noninvasive character, impedance spectroscopy was used for continuous monitoring of the progression of excised tissue samples during ischemia. We have developed a fast, noninvasive, automated method for quantitative analysis of impedance spectra of tissue samples, capable of revealing, through characteristic parameters (dispersion amplitudes, time constants and distribution parameters) membrane based microscopic processes like the closure of gap-junctions (a characteristic of the early alterations of ischemic tissues in the reversibility phase). Microscopic and equivalent circuit modeling was used to probe the effect of closure of cell connections and of changes in electrical properties of cell constituents on impedance spectra. We have developed a normalizing procedure emphasizing the pattern of ischemic alterations and enabling the comparison of different data sets.

Key words : alteration of membrane structure, ischema, impedance spectroscopy

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The interruption of the blood flow through a tissue or an organ, is globally known as the ischemic process or ischemia. Due to an impaired oxygenation and elimination of metabolic residues, ischemia is accompanied by major alterations of the morphology and composition of tissue constituents leading in time to loss of tissue function and finally to cellular death. Alterations of membrane structure and function are essential characteristics of cells undergoing ischemia or reoxygenation/reperfusion and include functional, compositional and fluidity changes and possible bleb formation (Herman *et al* 1988, Gores *et al* 1990, Matthews 1994). In final stage, cellular death is accompanied by cell membrane disintegration. Though the concept of viability is rather undefined at tissue scale, it is conceivable that for highly organized structures, both liver and heart tissues acting as functional syncitia, the lose of the functional integrity marks the "death" of the tissue. From resuscitation experiments (involving the shift back to the normal blood perfusion) it was proven that the limit of tissue viability, though dependent on the temperature, tissue type and energy reserves, is far earlier than any dramatic cellular changes. Therefore, in conjunction with the documented acidification of the intracellular space (McCord 1988, Taylor 1999) affecting the state of gap junctions (Tortora 1994, Bennett 1992, Goodenough 1992), alteration of the cell to cell communication via gap junctions has been identified as one of the possible processes taking place in the early phase of ischemia.

This study is mainly focused on quantitative evaluation of the progression of tissue state during ischemia, specifically in the domain of reversibility, as it is been revealed by impedance spectroscopy measurements.

Impedance spectroscopy enables the estimation of the electrical properties of a system (defined in terms of its ability to store - i.e. its capacitance C, and transfer charge - i.e. its conductance G) from its response to a sinusoidal applied perturbation over a pre-defined frequency range. The signal imposed (as either current or voltage) via two electrodes placed in contact with the probe determines the polarization of the sample and correspondingly a change in the amplitude and the phase of the measured signal. The impedance of the probe is calculated as the ration of the applied voltage and the current passing through the probe as a function of frequency ν:

$$Z = |Z| \cdot e^{j\phi} = Z' + j \cdot Z'' = \frac{V \sin(2\pi\nu)}{I \sin(2\pi\nu + \phi)}$$

Using equivalent electrical circuits or detailed microscopic models, from the real and imaginary part of impedance one can determine the electrical parameters of the different compartments of the system (a comprehensive description of the technique is given by Macdonald 1987).

Due to the lack in detailed microscopic models associated with the complexity of the ischemic tissue, the computational difficulties in conjunction with the parameter evaluation and last but not least the biological variability (dependence on e.g., tissue type and energy reserves), the characteristic evolution of tissue during ischemia has been usually followed using chosen frequencies and representing the "raw" (measured) data in the form of real or imaginary part of impedance.

Based on characteristic evolution of the real part of impedance at 200 Hz (Figure 1) and resuscitation experiments, three distinct domains have been identified: A) minor changes in tissue state and temperature equilibration (the excised tissue reaches the experimental temperature), B) significant changes of the spectra characterized by a steep increase of the amplitude of the dispersion up to a plateau value, the time duration (τ) of this domain, highly dependent on tissue state and incubation temperature, is critical for successful resuscitation procedures, C) loss of viability - though there are minor structural and morphological changes from that point on, resuscitation experiments proved that the tissue function can no longer be restored. At the end of this domain cell membrane alterations could occur determining a decrease in the measured values.

Using the analysis of complete impedance

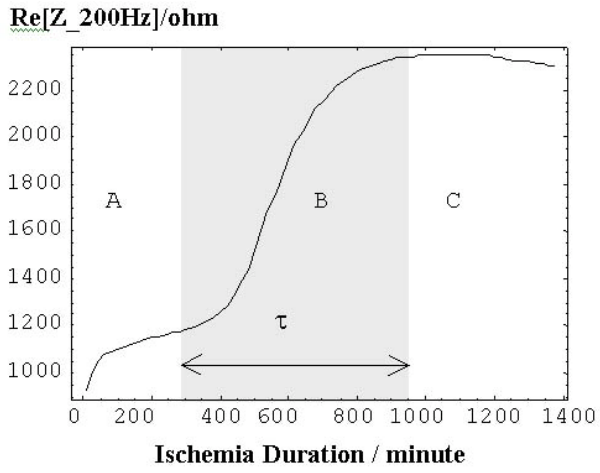


Figure 1. Characteristic time evolution of the measured real part of impedance at 200 Hz during ischemia displaying three characteristic regions of evolution:
A) minor changes in tissue state and temperature equilibration,
B) significant changes of the spectra characterized by a steep increase of the amplitude of the dispersion up to a plateau value, estimation of the time duration (τ) of this domain is critical for successful resuscitation of the organ
C) loss of tissue viability. Measurement were performed with a 15 minutes interval between acquisitions at 15 °C

spectra (simultaneous fitting of the measured real and imaginary parts of impedance with equivalent models) and theoretical modeling we specifically address the hypothesis that the closure of the gap junction is responsible for the evolution of the measured impedance while still in the reversibility domain.

Methodology

Due to it's capability to reveal inhomogeneities, as well as it's noninvasive character, impedance spectroscopy in a broad frequency range (1 Hz-20 MHz) was used for continuous monitoring of the progression of excised tissue samples during ischemia. The structure of the experimental set-up is presented in Figure 2 and includes thermostated chambers with 4 planar electrodes placed, in direct contact with the probe, on the chamber floor, and a computer controlled Solartron 1260 equipment for impedance determination.

Tissue samples were pig liver and dog heart (ventricle) subject to either "pure" ischemia (interruption of blood flow) or "perfused" ischemia (before the excision the whole organ was perfused with protective solutions designed to minimize the ischemic insult on the organ, among them HTK -in mmol/L: 15 NaCl, 9 KCl, 4 $MgCl_2$, 180 Histidine, 18 Histidine HCl, 30 manitol, 2 tryptophan, 1 K Ketoglutarate (Custodiol®) and St. Thomas-in mmol/L: 91.1 NaCl, 14.8 KCl, 15 $MgCl_2$, 1.2 $CaCl_2$, 25 $NaHCO_3$, 1.2 procaine HCl, 1.2 $MgSO_4$, 1.2 KH_2PO_4) were excised and placed on top of the electrode system, in incubated chambers. Due to the biological variability, simultaneous experiments were performed with tissue samples extracted from the same organ maintained in separate chambers at temperatures ranging from 5-35 °C. The spectra automatically recorded were displayed and analyzed off line.

The quantitative analysis of the impedance spectra in the frequency domain: 1 Hz ÷ 20 MHz involves nonlinear complex fitting of the data with phenomenological models (Havriliak-Negami equations (Böttcher and Bordeweijk 1978) with two time constants). To this purpose, modified algorithms enabling simultaneous fitting of both real and imaginary parts of complex immitances (impedance/admittance/dielectric constant) as well as data normalization, have been developed. The values of characteristic spectral parameters (dispersion amplitudes, characteristic time constants and distribution parameters) were derived for each spectrum (that contains 58 frequency points) in less than a minute, enabling complete quantitative description of the whole frequency domain.

Theoretical modeling of pairs of gap connected cells (Gheorghiu *et al*. 2002) involves an extension of the theory of dielectric behavior of non-spherical cells (Vrinceanu and Gheorghiu

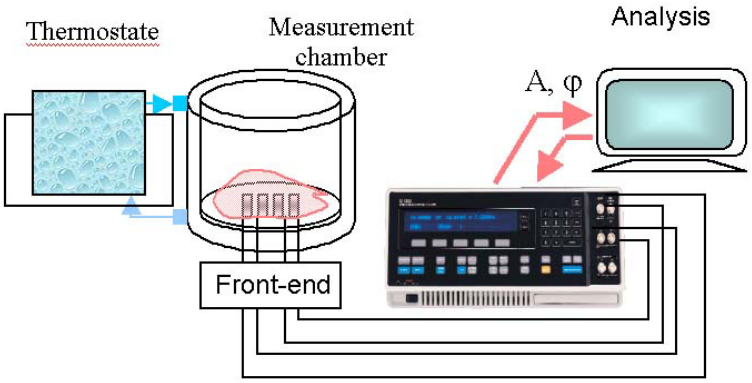


Figure 2. Experimental set-up enabling continuous measurement of excised tissue samples under controlled temperatures

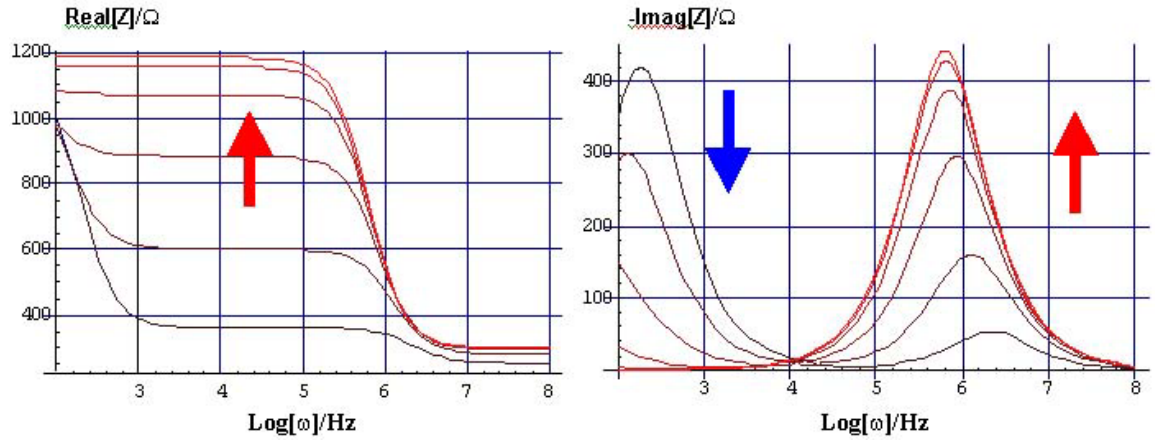


Figure 3. Simulation of the effect of closure of gap-junctions (an increased associated resistance) on the real and imaginary components of complex impedance. The arrows indicate the evolution of the two dispersions with the closure of gap junctions

1996) to the high-concentration domain. In comparison with the finite element method (for a comprehensive study one can consult Fear and Stuchly (1998)), which can be applied to intricate shapes but requires significant computing facilities, this approach is restricted to shapes with rotational symmetry, but on an ordinary PC it can provide in several minutes the dielectric (impedance) spectra of a suspension of particles exhibiting any orientation, cell concentration, number of shells or electric properties that can be easily chosen.

Results

Depicting the excised tissue as rows of interconnected cells in parallel to a conductive pathway (corresponding to the extracellular medium) and associating an equivalent circuit allows an intuitive representation of what could be the effect of the closure of gap junctions on the impedance spectra: an abrupt increase of the dispersion amplitude concomitant to a reduction (up to disappearance) of the amplitude of a low frequency dispersion (Figure 3).

Since the tissue non homogeneity and complexity (not encompassed by this simplistic model) determines broader and non symmetric spectra, the analysis was performed on using complex nonlinear fit routines on the basis of Havriliak Negami equation:

$$Z^{*} = LI + \frac{A_1}{\left[1+(i\cdot\omega\cdot\tau_1)^{1-\alpha_1}\right]^{\beta_1}} + \frac{A_2}{\left[1+(i\cdot\omega\cdot\tau_2)^{1-\alpha_2}\right]^{\beta_2}}$$

providing the limit at large frequencies (LI), the amplitudes (A_1, A_2), time constants (τ_1, τ_2) and distribution parameters (α_1, α_2, β_1, β_2,) for the two dispersions.

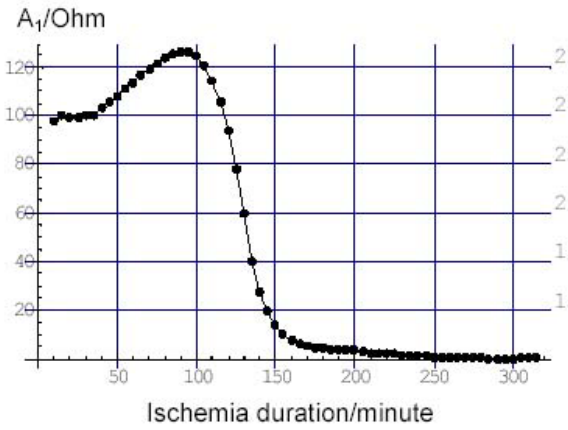
Only in the case of liver tissue using modified (sputtered with iridium) electrodes it was experimentally possible to detect two separate dispersions, with the low frequency one disappearing during ischemia (Gersing and Hofmann, 1995), Figure 4 revealing the derived amplitudes for the two measured dispersions at 10 Hz and 1.3 kHz respectively.

The intuitive picture of a decrease in equivalent capacitance of the system when the gap-junction communication is abolished (i.e. equivalent to membrane capacitors in series) is supported by the evolution of equivalent capacitances (Figure 5) derived from the measured data.

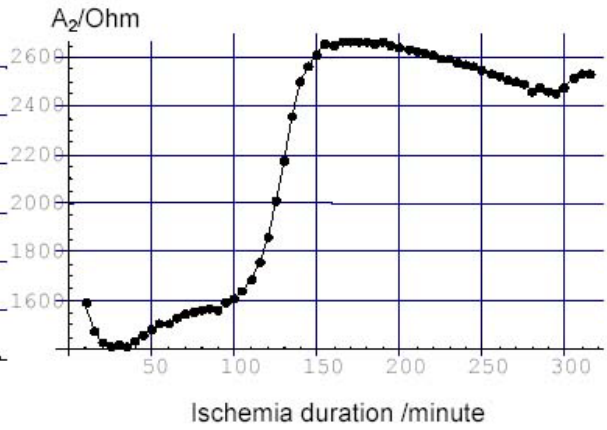
Unfortunately the experimental access to the low frequency domain, where significant changes have been observed can be problematic as is in the case of heart tissue, therefore our analysis was focused on the high frequency dispersion in an attempt to find suitable parameters to follow the evolution of both heart and liver ischemic tissues.

Despite the biological variability, given by morphological individuality, as well as the dependence on the "history" of the tissue, that makes the comparison of different experiments an intricate task, we have shown that time evolution of the distribution parameters of the high frequency dispersion, α_2, exhibits characteristic patterns during ischemia, as revealed in Figure 6, able to be used as an universal marker of the ischemic process.

Not just mathematical instruments, the distribution parameters are related with the sample inhomogeneity and act as relevant indicators of the way the current flows inside the tissue. Depending on the a.c. frequency, the current pathway (and related impedance behavior) is tuned by the state of membranous connections between the cells. Therefore, based on the moment when the distribution parameter reaches a peak, we have developed a normalization procedure:



Amplitude of the 10 Hz dispersion in the case of porcine liver tissue maintained at 25 °C



Amplitude of the main dispersion in the case of porcine liver tissue maintained at 25 °C

Figure 4. The evolution of the derived dispersion amplitudes during ischemia in the case of porcine liver

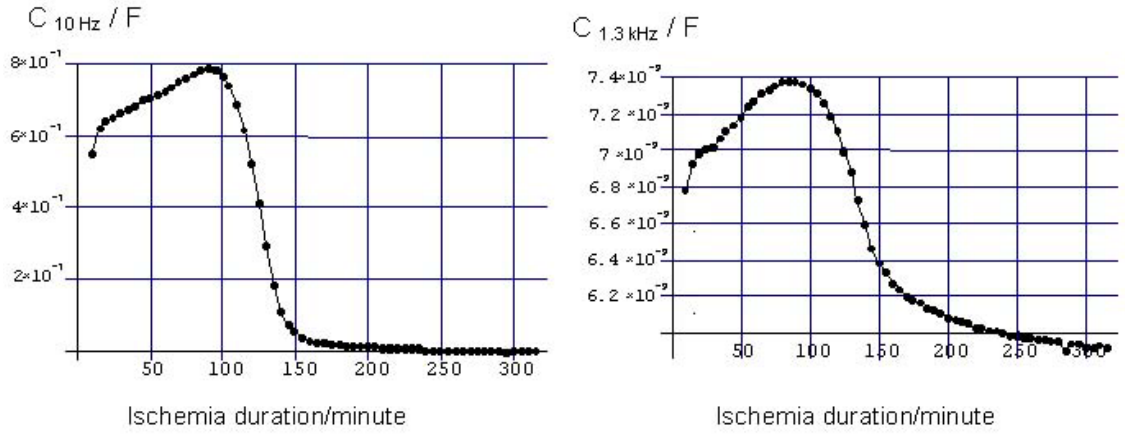


Figure 5. The evolution during ischemia of the derived equivalent capacitances (at low, 10Hz, and higher frequency, 1.3 kHz) in the case of porcine liver. It should be noted that with increasing frequency the change in capacitance values is less significant

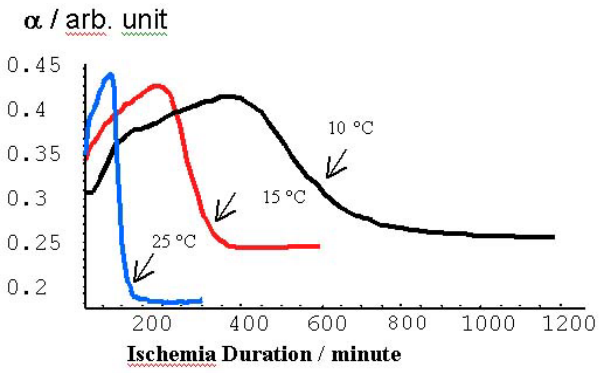


Figure 6. The time evolution of the distribution parameter of the main dispersion for several incubation temperatures

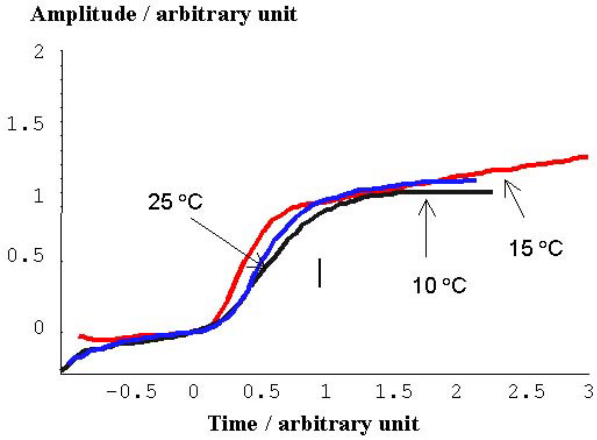


Figure 7. The normalized values of the dispersion amplitude for 3 distinct temperatures showing a similar pattern of evolution (and probably the same underlying mechanism of closure of gap - junction communication)

$$NX[t] = \frac{X[t] - X[t_{max}]}{X[t_{max}]}$$

where NX[t] stands for the normalized value while X[t] is the actual value of the derived spectral parameter to be normalized and $X[t_{max}]$ is the value corresponding to the same moment in which the distribution parameter reaches the peak value (Gheorghiu and Gersing 1999) that enabled us to compare experiments performed at various temperatures according to Figure 7.

The same procedure has been applied to processed data related to two protective solutions revealing clear separation between them and enabling a comparison of their protective capabilities.

The myocardial tissue is more inhomogeneous than the liver tissue and histology determinations (Schmiedl *et al*. 1996) prove a more pronounced effect of ischemia on mitochondria

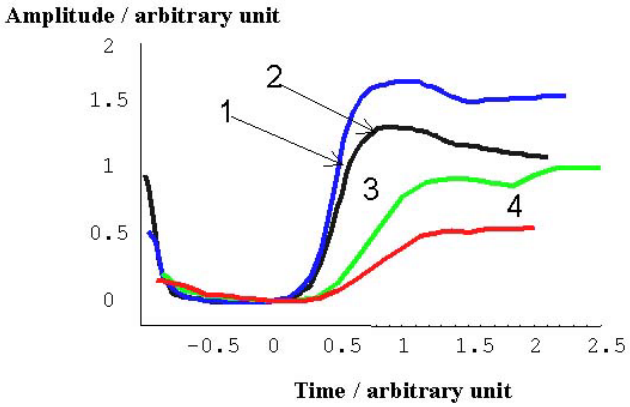


Figure 8. Paired experiments with St. Thomas protective solution (evolutions 1&2) and HTK (evolutions 3&4) revealing better protective capabilities of the HTK solution in connection to longer time intervals until the plateau values are reached

(e.g. mitochondria clearance and membrane disruption). This is revealed by impedance spectrum through a highly asymmetric dispersion during the early phase of ischemia (data not shown). While the ischemia progresses there is a steep increase in the amplitude of the beta dispersion and less and less heterogeneity of the tissue sample as revealed by the values of the derived asymmetry parameter β_2.

Several experiments have shown two separate dispersions at the beginning of the ischemic process proofing that asymmetric dispersions must be associated with a superposition of closely positioned distinct dispersions.

The microscopic model of pairs of interconnected cells (Gheorghiu *et al*. 2002) was used in both impedance and dielectric representations to test the effect of closure of cell connections and electrical properties of cell constituents on the related spectra. Though a rough approximation of the biological tissue (it involves pairs of cells and no organelles or tubules were modelled) our microscopic model provides a similar behaviour with the experimental one: the closure of the gap is reflected by an increase in the amplitude of the dispersion (in impedance terms) while in the permittivity there is a significant decrease of the plateau at low frequencies (in accordance with the data in (Figure 5).

Discussions and Conclusions

Tissue viability depends on the integrity of structural elements and on the effective ways of communication between the cells, targeting the attention towards the possible alterations of membranous structures during ischemia. While cell membrane disruption takes place hours after the onset of severe ischemia (Schmiedl *et al*. 1996), long after the tissue is dead, by using a custom-made fast, noninvasive, automated, method for quantitative analysis of impedance spectra and complex phenomenological models, we were able to reveal membrane based microscopic processes (i.e. the closure of gap-junctions) as characteristics of the early alterations of ischemic tissues in the phase of reversibility. Possible membrane alterations of mitochondria as revealed in experiments performed on heart tissue awaits further clarification in conjunction to a more detailed model taking into account the existence of extensive tubular system (Alberts *et al*. 1994) that might alter the way the current flows inside the tissue.

Computer simulations based on microscopic as well as phenomenological models suggest that the overall changes of impedance spectra are reflecting, on tissue scale, the effect of complex processes, like membrane permeabilization, closure of gap junctions and edema formation, running in parallel. Therefore, we stress on the necessity of considering the parametrisation of the whole spectrum for the proper analysis of the ischemic tissue.

We have shown the abilities of a normalizing procedure, based on the evolution of the distribution parameter connected to H-N phenomenological model, to provide an “internal” reference system for the ischemic process, eliminating the requirement for control experiments and enabling the comparison of different data sets.

Though performed on excised tissues the

procedure developed might be the starting point for a continuous monitoring system for clinical application.

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