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Changes in mitochondrial functioning with electromagnetic radiation of ultra high frequency as revealed by electron paramagnetic resonance methods

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Abstract

Purpose: **To study the effects of electromagnetic radiation (EMR) of ultra high frequency (UHF) in the doses equivalent to the maximal permitted energy load for the staffs of the radar stations on the biochemical processes that occur in the cell organelles.**

Materials and methods: **Liver, cardiac and aorta tissues from the male rats exposed to non-thermal UHF EMR in pulsed and continuous modes were studied during 28 days after the irradiation by the electron paramagnetic resonance (EPR) methods including a spin trapping of superoxide radicals.**

Results: **The qualitative and quantitative disturbances in electron transport chain (ETC) of mitochondria are registered. A formation of the iron-nitrosyl complexes of nitric oxide (NO) radicals with the iron-sulphide (FeS) proteins, the decreased activity of FeS-protein N2 of NADH-ubiquinone oxidoreductase complex and flavo-ubisemiquinone growth combined with the increased rates of superoxide production are obtained.**

Conclusions: **(i) Abnormalities in the mitochondrial ETC of liver and aorta cells are more pronounced for animals radiated in a pulsed mode; (ii) the alterations in the functioning of the mitochondrial ETC cause increase of superoxide radicals generation rate in all samples, formation of cellular hypoxia, and intensification of the oxide-initiated metabolic changes; and (iii) electron paramagnetic resonance methods could be used to track the qualitative and quantitative changes in the mitochondrial ETC caused by the UHF EMR.**

Keywords: Mitochondrial electron transport chain (ETC), ultra high frequency (UHV), electromagnetic radiation (EMR), superoxide, electron paramagnetic resonance (EPR)

Introduction

The influence of the electromagnetic radiation (EMR) of ultra high frequency (UHF) on biological processes attracts a great attention for many reasons. Firstly, it is governed by military applications. Secondly, the positive therapeutic effects of non-thermal and non-ionizing EMR in vivo and in vitro for bone repair, nerve and immune system stimulation, etc., are exploited for a long time (Pirogova et al. 2009), though their mechanisms are still poorly understood. Thirdly, because of the increasing number of mobile phone base stations, mobile communication systems and other devices operating in the range from radiofrequency (RF) up to the microwave frequencies (MW), their role in the environmental and occupational health risks are in a focus of public concern (Santini et al. 2003, Pirogova et al. 2009, Giuliani and Soffritti 2010). Fourthly, the colossal achievements in magnetic resonance (MR) methods to study biological relevant objects in vivo, in vitro and in situ demand high power levels of high RF and/or high MW frequencies. Modern commercially available nuclear magnetic resonance (NMR), electron paramagnetic resonance (EPR) spectrometers, their variations for imaging (nuclear magnetic resonance imaging (MRI), electron paramagnetic resonance imaging (EPRI) and double resonance techniques (Pulatova et al. 1989, Saifutdinov et al. 2001, Eaton et al. 2005, Abdul'yanov et al. 2008, Tsvetkov and Grishin 2009, Gafurov et al. 2010, 2012, 2013, Yavkin et al. 2012, Gafurov 2013, Kutin et al. 2013) are functioning in the range of 60 MHz–260 GHz both in continuous and pulsed modes and are able to provide up to 1 kW power even at high MW frequencies of 260 GHz by using short pulses (in nanoseconds range) sometimes applied to a tiny (less than 0.1 mm in diameter and 100 ng in mass) amount of the material studied (Gafurov et al. 2012, Biktagirov et al. 2013, Gafurov 2013). While thermal and ionizing effects of EMR are pretty well described and tracked in those experiments (Abdul'yanov et al. 2008, Gafurov et al. 2012, 2013, Yavkin et al. 2012, Gafurov 2013), the influence of the non-thermal and non-ionizing EMR on biological samples especially in the time-consuming research is practically totally unexplored. It is worth noting that the majority of the modern MR techniques are inherently pulsed techniques.

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All these motivate the scientific community to understand the mechanism(s) of interaction(s) between RF/MW radiation and biological systems. It goes hand-in-hand with a problem of a choice of an appropriate analytical tool(s) for that because the EMR may affect the cells at different levels of their structure: The surface receptors changing their distribution and conformation, the cellular membrane changing its rigidity and permeability, mitochondrial metabolic activity, transcription and translation processes or several of these elements at different intensities, etc. (Giuliani and Soffritti 2010).

In present work we studied the influence of continuous and pulsed UHF radiation on tissues of different organs of rats by the techniques of the conventional X-band (9 GHz) EPR (also denoted as ESR [electron spin resonance]) including a spin trapping of the generated superoxide ($O_2^{\bullet}$) radicals (Janzen 1971, Pulatova et al. 1989, Saifutdinov et al. 2001, Swartz et al. 2004, Eaton et al. 2005, Burlaka and Sidorik 2006b, Burlaka et al. 2013). EPR is well known as the most sensitive tool for the detection, identification and quantification of the naturally existing or artificially created (for example, by ionizing radiation) radicals (Pulatova et al. 1989, Eaton et al. 2010, Yavkin et al. 2012, Biktagirov et al. 2013, Burlaka et al. 2013, Gafurov et al. 2013). Different companies all over the world produce EPR spectrometers widely used in the biochemical, radiation and clinical laboratories. Even though, the capabilities of the method for the scientific purposes and for its clinical applications, in our opinion, are still not fully exploited. To our knowledge, we report the first comprehensive EPR study on the issue of the biological influence of the non-ionizing UHF EMR.

Materials and methods

Ninety-two mature male Wistar rats (1 month old) with a body mass of 200 ± 20 g from the vivarium of the R. E. Kavetsky Institute were chosen for the experiments. The maintenance and manipulations with animals were carried out according to the regulations of 'The basic ethical principles of experiments on animals' approved by the First National Congress of Bioethics of Ukraine (Kiev, 2001) and were guided by the statute and procedures of the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (Strasbourg, 1985).

The rats were divided into three groups. Animals of group I and II (each of 40 rats) were exposed to non-thermal UHF radiation. The animals of group I underwent the pulsed radiation from the 'Volna' generator ('EMA' Ltd, Moscow, Russia) operating at the frequency of 465 MHz with pulse modulation: The pulse duration – 2 ms; pulse period – 10 ms; total exposure time – 17.5 min; energy flux density (EFD) – 6.0 ± 2.4 mW/cm^2. Animals of group II were continuously irradiated during 15 min total time with the same EFD by the 'Luch-11' generator ('EMA' Ltd, Moscow, Russia) at the frequency of 2.45 GHz. Group III (12 intact animals) served as a reference group.

The value of EFD was chosen as corresponding to a maximal permitted energy load for the staff of the radar stations that is 1000 μW/cm^2 for the 300 MHz–300 GHz frequency range according to the norms of the Euro-Asian Council for Standardization, Metrology and Certification (EASC) (GOST 1999). We recalculated that value taking into account the differences between the humans and animals specific absorption rates and rates of the metabolic heat production for various frequency ranges (Durney et al. 1986). It resulted in the value of about 6.0 mW/cm^2 for the both carrying frequencies exploited in our research. We used an exposure in the quasi-anechoic (echo-free) screened chamber of 80×70 cm in size. A similar experimental exposure set-up was used by Kesari et al. (2012), for example. The spatial inhomogeneity of the electromagnetic field with linear polarization in the chamber was estimated to be less than 20%.

Teams of 10 rats from group I, 10 rats from group II and three rats from group III were devitalized by decapitation on days 1, 7, 14 and 28 after the irradiation for further post-mortal examination and preparation of samples for investigation *ex tempore*. The results were compared with those obtained on the samples of intact animals. The reliability of the obtained values was estimated according to Student's *t*-criterion.

A fixed amount of liver, heart or aorta tissues (500 mg) were placed into the positive mould and frozen down to the liquid nitrogen temperatures ($T = 77°C$) to get solid cylindrical samples with a length of 30 mm and a diameter of 4.5 mm. The samples were transferred in the quartz Dewar flasks directly into the EPR cavity of RE-1307 spectrometer (Experimental Institute of the Academy of Sciences of USSR, Chernogolovka, Moscow Region, Russia). The magnetic field (B0) created by the electromagnet of the spectrometer could be swept in the range of (10–600) mT (millitesla) equivalent to (100–6000) Gauss (G).

EPR measurements were conducted in continuous-wave (cw) mode at frequency of about 9 GHz (X-band) by using conventional modulation technique. The maximal microwave power applied during the measurement time of some minutes was 40 mW. The spectra were registered with the sweep rate of B0 of 1500 G/120 s and the modulation amplitude of 10 G at 100 kHz. The spectrometer handling, data collection and analysis were carried out with the home-made personal computer interface and self-written software. The EPR and the corresponding spin trapping measurements are described in details in (Burlaka et al. 2013).

The most important numerical characteristics of the EPR spectra are the positions (g-factor), the line widths and the intensities (amplitudes) of the lines of the EPR spectra (See Eaton et al. 2010 for the basics of the EPR research). In the present work we tracked only the amplitudes of some specific EPR lines because their positions and line widths remained practically unchanged.

Direct measurements of superoxide is usually problematic due to its short lifetime and its continuous detoxification by intra- and extracellular antioxidants. Oxidation of the 1-hydroxy-2,2,6,6-tetramethyl-4-oxo-piperidine hydrochloride (TEMPONE-H) to the stable nitroxyl radical 2,2,6,6-tetramethyl-4-oxo-piperidinoxyl (TEMPONE) under the influence of superoxide radical was used to trap the superoxide (Krinitskaya and Volodarskii 1982, Dikalov et al. 1997). TEMPONE-H was synthesized by L. A. Krinitskaya and L. S. Vartanyan (Semenov's Institute of Chemical Physics,

Russian Academy of Sciences, Moscow, Russia). A fixed amount of the investigated sample in the phosphate buffer solution was mixed at RT with 20 μl of the spin trapping solution (2 mg of the powder of TEMPONE-H dissolved in 10 ml of the phosphate buffer with pH = 7.4). EPR spectrum of the produced in this reaction stable radical TEMPONE consists of three lines due to the hyperfine interaction of the unpaired electron with the nitrogen nucleus and three possible projections (+ 1, 0, − 1) of a nuclear magnetic spin I = 1 of 14 N. The concentration rate of growth of the nitroxyl radical TEMPONE in the EPR mesh which corresponds to the generation rate of superoxide anion was measured at 2-min intervals via the change of the amplitude of the second hyperfine component of the EPR spectrum of TEMPONE during 6 min after the introduction of the mixture into the EPR cavity at RT. To minimize the hardware influence on the results, the B0 sweep rate of 100 G/120 s with the modulation amplitude of 0.2 G were chosen.

Results

EPR spectra of the liver, heart and aorta samples from the rats of group I, their changes with time and their comparison with the EPR spectra from the rats' tissues of reference group III are presented in Figures 1–3, respectively.

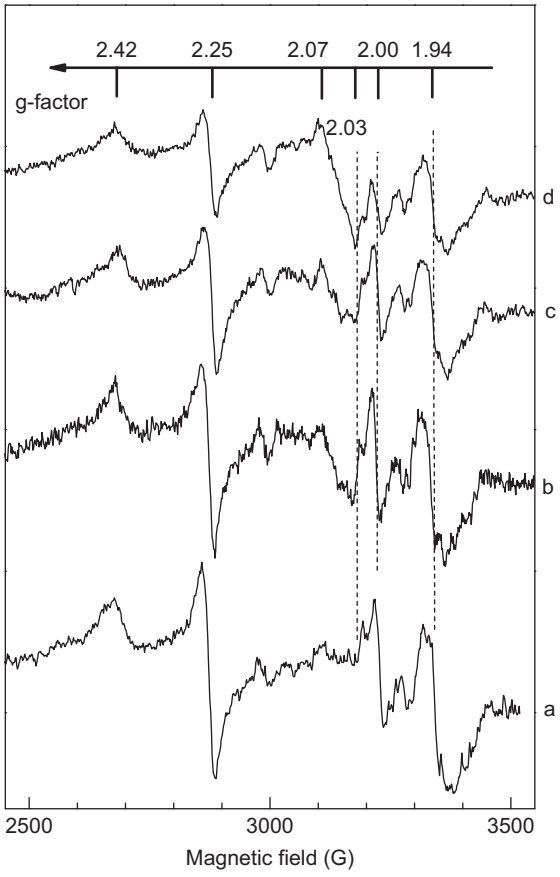

Figure 1. Liver cells EPR spectra of rats from groups III (a) and I (b–d) on the 1 (b), 7 (c) and 28 (d) days after the UHF influence. The vertical dashed lines are guides for eyes to mark the changes of the signals with g = 1.94; 2.00 and 2.03, respectively. The spectra are shifted vertically; the vertical scale is given in arbitrary units.

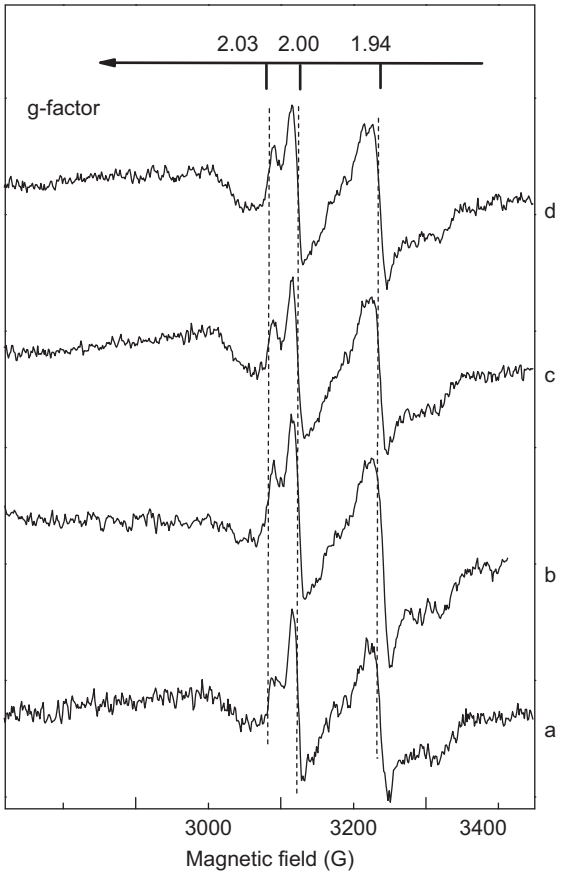

Figure 2. EPR spectra of the heart tissues. The vertical dashed lines are guides for eyes to mark the changes of the signals with g = 1.94; 2.00 and 2.03, respectively. The spectra are shifted vertically; the vertical scale is given in arbitrary units.

In liver cells, a decrease of the intensities of the signals with g = 1.94 and g = 2.00 combined with an increase of the intensity of the line at g = 2.03 were obtained from the first day of the observation (Figure 1). As measured on day 28, the amplitudes of these signals differed from those in a norm by almost three times ($p < 0.05$). Practically the same trend was registered for the EPR signals with g = 1.94; 2.00 for the rats of group II but their changes were found to be smaller (50% and less). The amplitude of the signal with g = 2.03 was practically the same as in a norm ($p < 0.05$). From these facts it could be immediately concluded that the modulated UHF EMR affects the liver cells much stronger than the continuous one.

In cardiomyocytes, only insignificant variations of the EPR spectra for groups I (Figure 2) and II (not shown) in comparison to reference group III were observed. Drastic changes were detected in the aorta samples, especially in group I (Figure 3). The intensity of the signal with g = 1.94 decreased immediately to 31 ± 3% (day 1, $p < 0.05$) and reached the value of 22 ± 3% on day 28 in comparison to that of reference group III (100%). Practically the same values were registered for the rats from group II. The level of flavo-ubisemiquinones (g = 2.00) for group I, in contrast, increased up to 200% (day 7, $p < 0.05$) and then remained practically unchanged. For the samples from group II, a reduction to the level of 40 ± 10% on day 1 that recovered only to the value of 68 ± 7% as compared

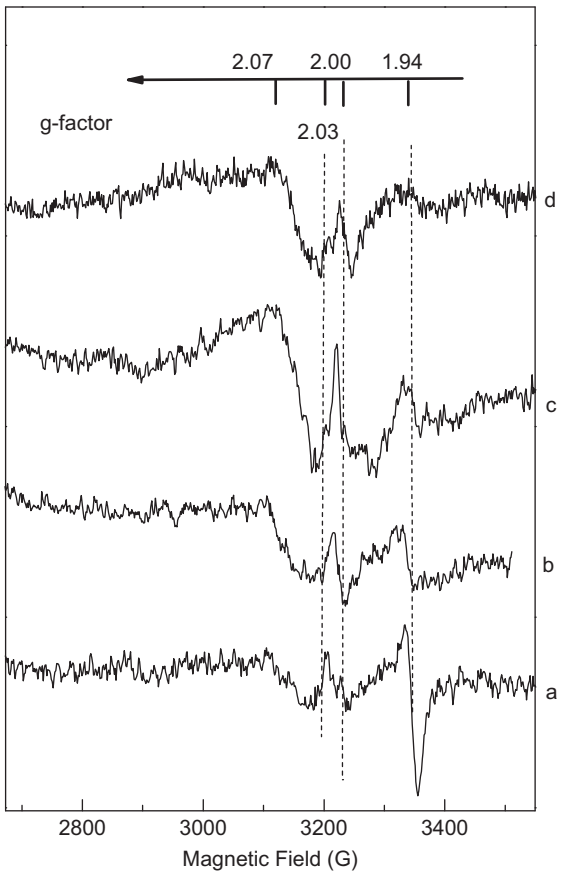

Figure 3. EPR spectra of the aorta tissues. The vertical dashed lines are guides for eyes to mark the changes of the signals with g = 1.94; 2.00 and 2.03, respectively. The spectra are shifted vertically; the vertical scale is given in arbitrary units.

to the control group were observed. That is the evidence of damage of the Q-cycle (an oxidation-reduction loop mechanism, see Osyczka et al. 2005, for example, for details) in the ubiquinone-cytochrome *p*-reductase of the mitochondrial electron transport chain (ETC). The signal at g = 2.03 for the samples of group I started to grow from day 7 and reached a value of 275 ± 22% on day 28 ($p < 0.05$). No statistically significant changes of the EPR signal with g = 2.03 were registered for group II samples.

Our measurements of the superoxide generation rates in the organs studied for all the animals groups are presented in Figure 4 (a–c). Dramatic increase (up to 9 times, $p < 0.005$) was observed from the first day of the experiment for the cells in all the organs studied. These rates did not reach their normal values even after 28 days of observation.

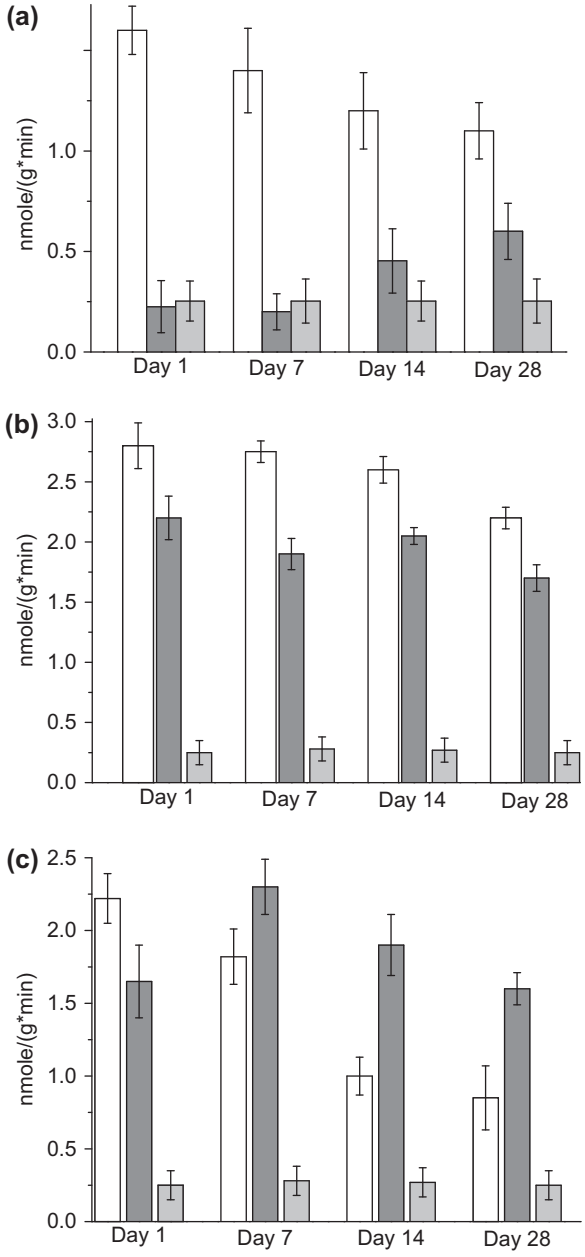

Figure 4. (a–c) Comparison of superoxide generation rates in mitochondria of liver tissue (a), of cardiomyocytes (b) and of the aorta samples (c) expressed in millimole per gram of tissue in minute after UHF influence and their changes with time for all the investigated groups. White, gray and light gray bars match to groups I, II and III, respectively. Error bars indicate the standard error of the mean value (SEM) for 10 (for groups I and II) or for three (results for group III) independent experiments.

Discussion

In the literature, the EPR signal at g = 1.94 is usually ascribed to the the iron-sulfur cluster N2 of NADH: (ubi)quinone oxidoreductase, also called respiratory complex I (Mallard and Kent 1969, Pulatova et al. 1989, Burlaka and Sidorik 2006b, Burlaka et al. 2013). N2 is believed to be the electron donor for (ubi)quinone as it is the most distal in the chain of the FeS clusters in this largest and is the most complicated enzyme of the mitochondrial ETC. The signal with g = 2.00 is usually ascribed to the free radical centers practically completely localized in mitochondria – semiquinones of flavoproteides found in the inner membrane of mitochondria and coenzyme Q semiquinones (ubisemiquinones) (Konstantinov and Ruuge 1977, Emanuel 1980, Burlaka and Sidorik 2006b). Formation of the complexes of the nitric oxides with the iron-sulfide proteins (NO-FeS complexes) in the mitochondrial ETC of N-type could be tracked through the changes of the signal with g = 2.03 (Pulatova et al. 1989, Burlaka and Sidorik 2006b, Burlaka et al. 2013).

As can be seen from the above, the changes in the EPR spectra obtained in the experiments are connected with the changes in the mitochondrial ETC. Mitochondria is known

as playing a key role in many human diseases related to their functions in cellular energy production, biosynthesis of essential cellular compounds, and involvement in autophagy and/or apoptosis regulation (Elliott and Head 2012). Some results, concerning cancer-related and radiation-induced mitochondrial dysfunctions are presented in our works (Sidorik and Burlaka 2000, Burlaka et al. 2006a, 2013, Burlaka and Sidorik 2006b).

The intensive spectral features in liver samples at g = 2.25 and 2.42 are ascribed to the heme-containing enzymes cytochrome *p*-450 (CYP) in membranes of the endoplasmic reticulum (microsomes) that represent a major part of the detoxification systems (Mallard and Kent 1969, Peisach and Blumberg 1970, Pulatova et al. 1989, Sidorik and Burlaka 2000, Saifutdinov et al. 2001). Detailed investigation of the dynamics of those is a matter of the ongoing experiments and is not in the scope of the present research.

As can be concluded from the EPR spectra, the heart cells are less sensitive to the effect of the UHF EMR (both in the pulsed and in the continuous modes) in comparison to the liver and aorta cells. The validity of this preliminary conclusion is discussed below along with the results of the spin trapping experiments.

Changes in the mitochondrial functioning in pathological processes are very often connected with the uncontrolled changes of the level of the reactive oxygen species (ROS) (Pulatova et al. 1989, Burlaka and Sidorik 2006b, Guthrie et al. 2008, Elliott and Head 2012, Burlaka et al. 2013). At physiological levels, ROS function as signaling molecules through oxidation of redox-sensitive cysteine residues and resultant inhibition of phosphatases that negatively regulate signaling cascades. The main source of cellular ROS is the mitochondrial ETC, through which continuous aerobic respiration generates $O_2^{\bullet-}$. ROS are thought to be the central elements of oxidative stress that could damage (directly or indirectly) important biological molecules, including DNA (Kim and Rhee 2004, Elliott and Head 2012).

Difference in the absolute values of the superoxide generation rates could be ascribed to the different total amounts of mitochondria in the tissues studied. Cardiomyocytes have the highest amount of those. This could explain high superoxide generation rates but very small changes in the EPR spectra (*cf.* Figure 2). Detailed investigation of changes in ETC in cardiomyocytes by using more sensitive EPR techniques are planned.

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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