UNIVERZITA PALACKÉHO V OLOMOUCI

PŘÍRODOVĚDECKÁ FAKULTA

KATEDRA BIOFYZIKY

BAKALÁŘSKÁ PRÁCE

DETEKCE UHLÍKOVÝCH RADIKÁLŮ V SRDEČNÍCH MITOCHONDRIÍCH POMOCÍ ELEKTRONOVÉ PARAMAGNETICKÉ REZONANČNÍ SPEKTROSKOPIE

Autor:	Vendula Paculová
Vedoucí práce:	doc. RNDr. Pavel Pospíšil, Ph.D.
Rok:	2018
Studijní obor:	Biofyzika

PALACKÝ UNIVERSITY OLOMOUC

FACULTY OF SCIENCE

DEPARTMENT OF BIOPHYSICS

BACHELOR THESIS

DETECTION OF CARBON-CENTERED RADICALS IN HEART MITOCHONDRIA USING ELECTRON PARAMAGNETIC RESONANCE SPECTROSCOPY

Author:	Vendula Paculová
Supervisor:	doc. RNDr. Pavel Pospíšil, Ph.D.
Year:	2018
Study programme:	Biofyzika

Bibliografická identifikace

Jméno a příjmení autora: Vendula Paculová

Název práce: Detekce uhlíkových radikálů v srdečních mitochondriích pomocí elektronové paramagnetické rezonanční spektroskopie

Typ práce: bakalářská

Pracoviště: katedra biofyziky

Vedoucí práce: doc. RNDr. Pavel Pospíšil, Ph.D.

Rok obhajoby práce: 2018

Abstrakt:

Bakalářská práce byla zaměřena na detekci uhlíkového radikálu v mitoplastech a submitochondriálních membránách pomocí elektronové paramagnetické rezonanční spektroskopie. Elektronový transportní řetězec buněčného dýchání lokalizovaný na vnitřní membráně mitochondrií je spojen s tvorbou reaktivních forem kyslíku. Tyto reaktivní formy kyslíku mohou oxidovat různé biomakromolekuly, čímž může docházet k tvorbě uhlíkového radikálu. Cílem práce bylo detekovat uhlíkový radikál pomocí vychytávače elektronů 5,5-1-pyrroline N-oxid (DMPO) a lokalizovat jeho tvorbu. V mitoplastech a submitochondriálních membránách izolovaných z vepřového srdce jsme detekovali uhlíkový radikál a určili místo jeho vzniku jako komplex I elektronového transportního řetězce.

Klíčová slova: uhlíkový radikál, mitochondrie, EPR, elektronový transportní řetězec, DMPO

Počet stran: 32

Počet příloh: 0

Jazyk: anglický

Bibliographical identification

Author's first name and surname:	Vendula Paculová
Title of thesis:	Detection of carbon-centered radicals in heart mitochondria using electron paramagnetic resonance spectroscopy
Type of thesis:	bachelor
Department:	Department of biophysics
The year of defence:	2018

Abstract:

The bachelor thesis was focused on the detection of carbon-centered radical in mitoplasts and submitochondrial particles using electron paramagnetic resonance spectroscopy. Electron transport chain of the cellular respiration localized in the inner membrane of mitochondria is associated with the formation of reactive oxygen species. These reactive oxygen species can oxidize various biomacromolecules, which leads to the carbon-centered radical formation. The aim of this thesis was to detect carbon-centered radical using spin-trap 5,5-1-pyrroline N-oxide (DMPO) and localized its formation. We detected carbon-centered radical in mitoplasts and submitochondrial particles isolated from pork heart and established complex I of electron transport chain as a source of the carbon-centered radical.

Key words:	carbon-centered radical, mitochondria, EPR, electron transport chain, DMPO
Number of pages:	32
Number of attachments:	0
Language:	English

Prohlašuji, že jsem bakalářskou práci na téma „Detekce uhlíkových radikálů v srdečních mitochondriích pomocí elektronové paramagnetické rezonanční spektroskopie“ vypracovala samostatně za použití v práci uvedených pramenů a literatury.

V Olomouci dne ……. ……

I declare that I developed individually the bachelor thesis titled “Detection of carbon-centered radicals in heart mitochondria using electron paramagnetic resonance spectroscopy” with showing all the sources and references.

In Olomouc ……. ……

Ráda bych touto cestou vyjádřila poděkování doc. RNDr. Pavlu Pospíšilovi, Ph.D. za jeho cenné rady a trpělivost při vedení mé bakalářské práce. Rovněž bych chtěla poděkovat Mgr. Ursule Ferretti za vstřícnost a pomoc v experimentální části bakalářské práce. Tato práce byla podporována studentským grantem z Univerzity Palackého (IGA_PrF_2018_022).

I would like to thank to doc. RNDr. Pavel Pospíšil, Ph.D. for his guidance and help during the preparation of my bachelor thesis. I would like to thank to Mgr. Ursula Ferretti for her helpful advices during the preparation of the experimental part of the thesis. This work was supported student grant from Palacký University (IGA_PrF_2018_022).

List of abbreviation

ATP	Adenosine triphosphate
CAT	catalase
DMPO	5,5-Dimethyl-1-pyrroline *N*-oxide
DNA	deoxyribonucleic acid
EDTA	ethylendiaminetetraacetic acid
ETC	electron transport chain
$FADH_2$	flavin adenine dinucleotide
$FADH^{\bullet}$	flavin adenine dinucleotide semiquinone
FET	forward electron transport
FMN	flavin mononucleotide
H_2O_2	hydrogen peroxide
$HO^{\bullet}$	hydroxyl radical
$HO_2^{\bullet}$	hydroperoxyl radical
HOCl	hypochlorous acid
KO_2	potassium superoxide
KOH	potassium hydroxide
MOPS	3-(N-morpholino) propanesulfonic acid
MTs	mitoplasts
NaCl	sodium chloride
NADH	nicotinamide adenine dinucleotide
NADPH	nicotinamide adenine dinucleotide phosphate
$^1O_2$	singlet oxygen
O_3	ozone

$O_2^{\bullet -}$	superoxide anion radical
Q	ubiquinone
QH_2	ubiquinol
$R^{\bullet}$	carbon-centered radical
RET	reverse electron transport
RH	alkane
RNA	ribonucleic acid
$RO^{\bullet}$	alkoxyl radical
$RO_2^{\bullet}$	peroxyl radical
ROS	reactive oxygen species
SMPs	submitochondrial particles
SOD	superoxidu dismutase
$SQ^{\bullet}$	ubisemiquinone
tRNA	transfer ribonucleic acid

Content

1. Introduction

This bachelor thesis is focused on the detection of carbon-centered radical in heart mitochondria. Mitochondria are cell organelles, which play an important role in the cellular respiration. Cellular respiration is composed of four steps including aerobic glycolysis, Krebs cycle, electron transport chain and oxidative phosphorylation Electron transport chain of cellular respiration localized in the inner membrane of mitochondria is associated with the formation of reactive oxygen species. Electron transport chain involves four enzymatic complexes and ATP synthase. The main sources of reactive oxygen species are complex I and complex III.

Reactive oxygen species are molecules with one, two or three oxygen atoms. They are divided into radical and nonradical. Radical reactive oxygen species include unpaired electron, while nonradical have no unpaired electron. In biological systems, reactive oxygen species produced as a byproduct of the metabolism play an important role in the cell signaling and homeostasis. However, overproduction of reactive oxygen species can lead to an oxidative stress. Oxidation of biomacromolecules such as proteins and lipids by reactive oxygen species leads to the formation of carbon-centered radicals. Oxidative stress is involved in the development of many diseases e. g. cancer, Parkinson's disease, Alzheimer's disease, atherosclerosis, vitiligo etc.

The lifetime of reactive oxygen species is very short ranging from nanoseconds to second. Electron paramagnetic resonance spectroscopy utilizing spin traps was used to detect radicals. These spin traps react with radicals to make more stable radical adduct, which we are able to detect.

2. Theory

2.1 Molecular oxygen

Molecular oxygen is a diatomic molecule consisting of two atoms of oxygen bound with a double covalent bond. It is diradical with two unpaired electrons located on different oxygen atom (Moss *et al*. 1995). Molecular oxygen has the triplet state as the ground state, whereas most of molecules are in the singlet state. The singlet state means that a molecule has electrons with antiparallel spin in orbital, while a molecule in triplet state has electrons with parallel spin. Due to spin orientation, molecular oxygen is nonreactive in its ground state. If molecular oxygen passes to the singlet state, it becomes strong oxidant.

2.2 Reactive oxygen species

Reactive oxygen species (ROS) are molecules consisting of one, two or three oxygen atoms. They can be divided into a radical and a nonradical ROS. The radical ROS consist of one or two unpaired electrons on the oxygen atom, whereas nonradical ROS have no unpaired electrons. Due to the presence of unpaired electrons, radical ROS are more reactive. In nature, ROS are formed as secondary products of the metabolism and play an important role in cell signaling and homeostasis (Devasagayam *et al*. 2004). Reactive oxygen species can be found in all aerobic cells, where thay are in balance with antioxidants (Waris, Ahsan 2006). On the other hand, overproduction of ROS can cause oxidative stress, which is involved in many diseases (Pham-Huy *et al*. 2008).

2.2.1 Classification of reactive oxygen species

Reactive oxygen species are classified according to electron configuration as radical and nonradical and according to formation as formed by energy transfer or formed by electron transport. Superoxide anion radical ($O_2^{\bullet -}$) and its protonated form hydroperoxyl radical ($HO_2^{\bullet}$), hydoxyl radical ($HO^{\bullet}$), peroxyl radical ($RO_2^{\bullet}$), and alkoxyl radical ($RO^{\bullet}$) belong to radical ROS, whereas hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$), hypochlorous acid (HOCl) and ozone (O_3) are non-radical ROS. There are two types of ROS according to formation. Reactive oxygen species formed by electron transport ($O_2^{\bullet -}$, H_2O_2, $HO^{\bullet}$) and by energy transfer is ($^1O_2$).

2.2.2 Characterization of reactive oxygen species

2.2.2.1 Superoxide anion radical

Superoxide anion radical is formed by one electron reduction of O_2 (Halliwell, Gutteridge 2015). It is a molecule containing one unpaired electron.

Due to the electron configuration, $O_2^{\bullet -}$ acts as both an oxidant and a reductant. Superoxide anion radical accepts electrons e^- (1) as an oxidant

$$O_2^{\bullet -} + Fe^{2+} \rightarrow H_2O_2 + Fe^{3+}. \quad (1)$$

and donates electrons e^- as a reductant (2)

$$O_2^{\bullet -} + Fe^{3+} \rightarrow O_2 + Fe^{2+}. \quad (2)$$

Superoxide anion radical is a base, which means, that $O_2^{\bullet -}$ has an ability to accept a proton H^+. The protonated form $HO_2^{\bullet}$ is formed by accepting a proton. This protonated from is more lipophilic and due to that it can be more toxic for membranes (Blake *et al*. 1987). Hydroperoxyl radical is the dominant form in acidic pH, whereas $O_2^{\bullet -}$ is the dominant form in neutral and alkaline pH. The reactivity of $O_2^{\bullet -}$ depends on a solvent, temperature and pH. The pK_a of the reaction

$$HO_2^{\bullet} \rightleftarrows H^+ + O_2^{\bullet -} \quad (3)$$

is 4.8, which means, that the ratio of $O_2^{\bullet -}$ and $HO_2^{\bullet}$ at pH 4.8 is 1:1 (Halliwell, Gutteridge 2015).

The standard redox potential of redox couple $O_2/O_2^{\bullet -}$ at pH 7 is $E_0' = -160$ mV. But this redox potential is only relevant if the concentrations of O_2 and $O_2^{\bullet -}$ are equal. In biological samples, the concentration of O_2 (250 mM) highly exceeds the concentration of $O_2^{\bullet -}$(hundreds nM). The redox power required for reduction of O_2 is determined by Nerst equation (4)

$$E = -0{,}16 + 0{,}06\log[0_2/0_2^{\bullet -}], \quad (4)$$

when the final redox power is 0 mV and more.

The lifetime of $O_2^{\bullet -}$ is μs to ms. Because of that short lifetime and low solubility in fats, $O_2^{\bullet -}$ can diffuse only for short distances (Blake *et al*. 1987).

2.2.2.2 Hydrogen peroxide

Hydrogen peroxide is a molecule which consists of four atoms with no unpair electron. It is a weak acid, which means, it has an ability to lose a proton H^+. Hydrogen peroxide is dissociated to hydroperoxide anion (HO_2^-) and a proton H^+ (7).

$$H_2O_2 \rightleftarrows H^+ + HO_2^- \quad (7)$$

Hydrogen peroxide acts as an oxidant and a reductant depending on pH. In the acidic environment, H_2O_2 is one of the strongest oxidants. It oxidizes ferrous ion (Fe^{2+}) to ferric ion (Fe^{3+}). This reaction is called the Fenton reaction (8).

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + HO^{\bullet} + OH^- \quad (8)$$

In the alkaline environment, H_2O_2 acts as a weak reductant. It reduces hypochlorite anion (OCl^-) to chloride anion (Cl^-), while $^1O_2$ and water are formed (9).

$$H_2O_2 + OCl^- \rightarrow {}^1O_2\left({}^1\Delta_g\right) + Cl^- + H_2O \quad (9)$$

Hydrogen peroxide is thermodynamically unstable and it spontaneously decomposes to water and molecular oxygen (10).

$$2H_2O_2 \rightarrow 2H_2O + O_2 \quad (10)$$

The increase of temperature, concentration and pH can speed up the decomposition of H_2O_2.

The lifetime of H_2O_2 is ms to s. Due to this long lifetime, H_2O_2 can diffuse inside or outside the cell.

Hydrogen peroxide is formed in human and animal cells as a product of biochemical processes. Hydrogen peroxide is toxic because of the peroxide ion (O_2^{2-}), might oxidizes proteins, membrane lipids and DNA (Löffler, Petrides 1988). Hydrogen peroxide is dangerous because it serves as substrate for $HO^{\bullet}$ formed by Fenton reagent.

2.2.2.3 Hydroxyl radical

Hydroxyl radical is a diatom molecule with one unpaired electron on oxygen atom. It is a highly reactive molecule with a short lifetime.

Hydroxyl radical is a strong oxidant known to oxidizes biomolecules (RH).

The standard redox potential of redox couple $HO^{\bullet}/H_2O$ at pH 7 is $E_0' = 2{,}3$ V. This redox potential allows to reduce $HO^{\bullet}$ to water easily. It is related to $HO^{\bullet}$ very short lifetime, which is in ns. Due to its very short lifetime, diffusion of $HO^{\bullet}$ is limited. It is generally considered that $HO^{\bullet}$ oxidizes biomolecules at the site of its formation.

2.2.4 The production of superoxide anion radical in mitochondrial membrane

Mitochondria are membrane organelles present in most eukaryotic cells. It consists of outer membrane, intermembrane space, inner membrane and matrix. The outer membrane is composed of phospholipid bilayers and large amount of integral membrane proteins called porins. These porins create channels, which allow molecules with molecular weight of 5 kDa and less to diffuse from the one side of membrane to the other (Alberts *et al.* 2002). Disruption of the outer membrane release proteins from the intermembrane space to the cytosol. This process leads to cell death (Chipuk *et al*. 2006).

The intermembrane space is permeable for small molecules. Thanks to that, a concentration of these molecules in the mitochondria is the same as a concentration in cytosol (Alberts *et al.* 2002).

The inner membrane contains cristae, which expand the surface of the inner membrane. It also contains about one fifth of the overall number of proteins in mitochondria (Alberts *et al.* 2002). Inner membrane also includes enzymes of the respiratory chain and the ATP synthase, which is a protein complex able to synthase ATP. Electron transport chain (ETC) is associated with ROS formation.

Matrix contains two third of overall number of proteins in mitochondria (Alberts *et al.* 2002). It contains ribosomes, tRNA, mitochondrial DNA and a large amount of enzymes. The main function of these enzymes is oxidation of pyruvate, fatty acids and take part in Krebs cycle, in which NAD+ is reduced to NADH and FAD is reduced to $FADH_2$ (Alberts *et al.* 2002).

Electron transport chain (ETC) is composed of four enzymatic complexes and the ATP synthase. These four complexes make a proton gradient, which drives the synthesis of the ATP (Alberts *et al*. 2002). However, during this process, $O_2^{\bullet -}$ is created especially in complex I and complex III.

Electrons from NADH and $FADH_2$, which are formed in the Krebs cycle, pass through four complexes to O_2. Molecular oxygen is then reduced to water.

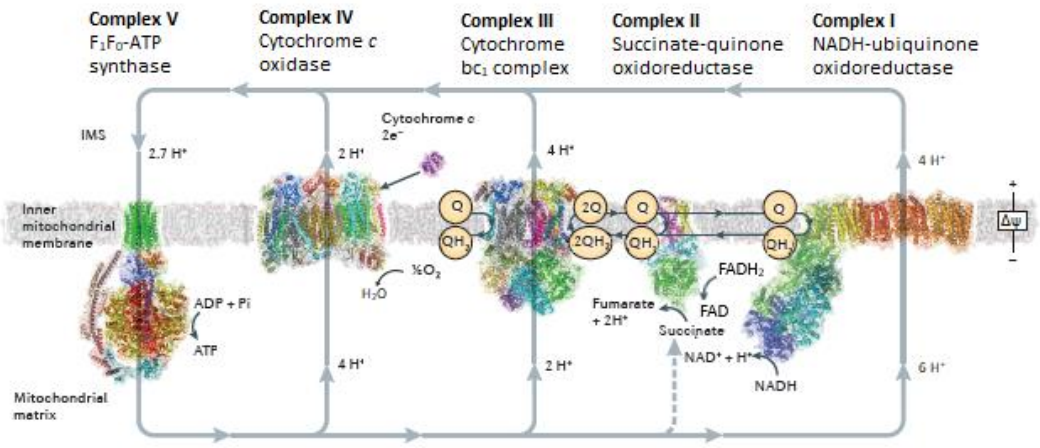

Figure 1 The electron transport chain. (Sazanov 2015)

2.2.4.1 Complex I

Complex I (NADH-ubiquinone oxidoreductase) is the first enzyme of ETC. It is the largest and the most complicated enzyme of ETC (Brandt 2006). Complex I catalyze a reaction

$$\mathrm{NADH} + \mathrm{H^+} + \mathrm{Q} + 4\mathrm{H^+_{in}} \rightarrow \mathrm{NAD^+} + \mathrm{QH_2} + 4\mathrm{H^+_{out}} \qquad (13)$$

where Q is coenzyme Q_{10} (ubiquinone), NAD^+ is oxidized form of NADH and QH_2 (ubiquinol) is reduced form of Q. Complex I is comprised of two parts. The first part is a hydrophilic arm at the matrix side, where flavin mononucleotide (FMN), iron-sulfur clusters (Fe-S) and Q are localized. The second part is a hydrophobic arm localized in the inner membrane. Complex I receive NADH from the Krebs cycle and it binds to the top of the hydrophilic arm (Figure 2).

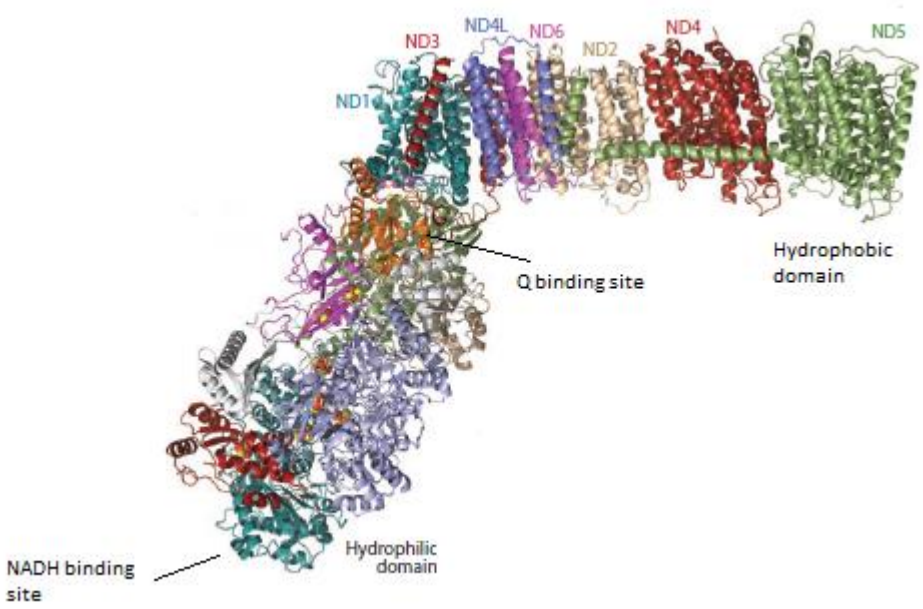

Figure 2 The structure of complex I with NADH binding site and Q binding site. (Hirst 2013)

At first NADH gives two electrons to the FMN and reduces it to $FMNH_2$. Then electrons pass though the Fe-S clusters to the Q binding site to reduce Q to QH_2. This process results in the release of four protons H^+ from the matrix to the inner membrane space (Figure 3) (Voet D. J. *et al.* 2012). Redox centers of complex I, which are involved in transport of two electrons from NADH to Q comprises FMN, eight Fe-S clusters and Q (Brand 2010).

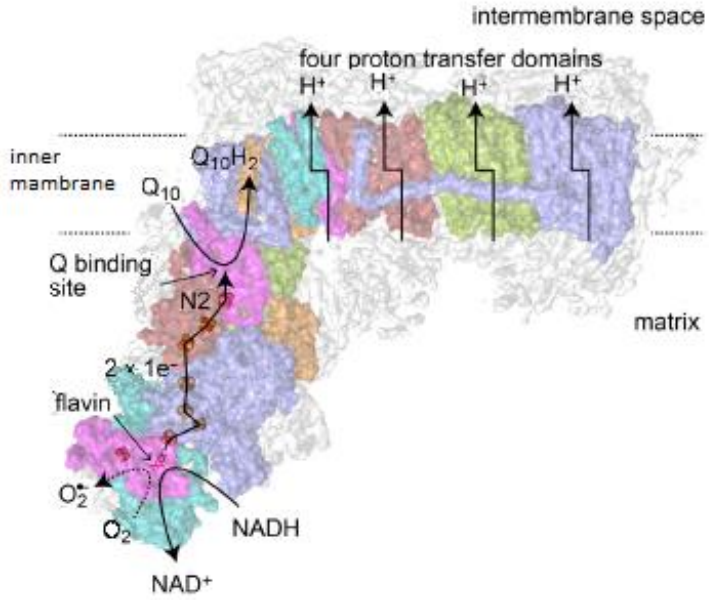

Figure 3 The transport of two electrons from NADH to Q and the transport of four protons from matrix to inner membrane space. (Hirst, Roessler 2015)

2.2.4.1.1 The production of superoxide anion radical in complex I

Superoxide anion radical can be produced during ETC in complex I. There are two pathways for $O_2^{\bullet -}$ production comprising the forward electron transport (FET) and the reverse electron transport (RET). Forward electron transport produces only a very small amount of $O_2^{\bullet -}$, less than 0,1% of the overall electron flow (Hansford *et al.* 1997), whereas more significant amount (up to 5% of electrons) contributes to $O_2^{\bullet -}$ formation during RET.

Forward electron transport is a process, where O_2 is reduced by $FMNH_2$. This process depends on the NADH/NAD^+ ratio. The redox potential of NAD^+/NADH is E_m = -320 mV and of $FMN/FMNH_2$ is E_m = -300 mV, which means that NADH is stronger reductant. The production of $O_2^{\bullet -}$ is the highest when the NADH concentration is much higher than the concentration of the NAD^+ (Kussmaul, Hirst 2006). This also explains why the inhibition of complex I by the complex I inhibitor rotenone increases $O_2^{\bullet -}$ production. It is well known that rotenone known to bind to the Q binding site and blocks the transport of electrons from Fe-S cluster N2 to Q. Then electrons return to the FMN and reduce it to $FMNH_2$ which produce $O_2^{\bullet -}$(Takeshige, Minakami 1979) (Figure 4).

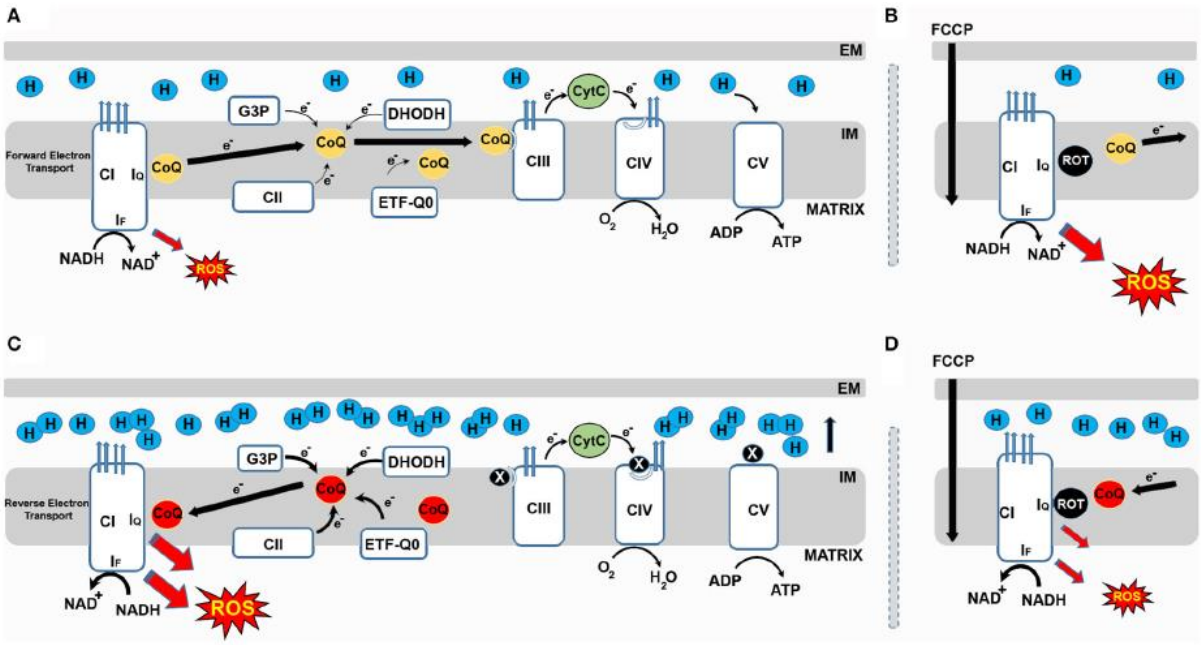

Figure 4 The production of the superoxide anion radical in the complex I. (A) Forward electron transport. (B) The inhibition of FET by the rotenone. (C) Reverse electron transport. (D) The inhibition of RET by the rotenone. (Scialò et al. 2017)

The production of the $O_2^{\bullet-}$ by the RET needs over-reduced Q pool and high membrane potential which lead to the reverse transfer of electrons from QH_2 to the complex I where these electrons pass through Fe-S clusters to the FMN (Figure 4). Flavin mononucleotide is then reduced to $FMNH_2$. Consequently, O_2 is reduced to $O_2^{\bullet-}$ as in FET. However, inhibition of the RET by rotenone decreases the amount of $O_2^{\bullet-}$.

2.2.4.2 Complex II

Complex II (succinate-quinone oxidoreductase) is the second enzymatic complex of the ETC. It is the only enzyme which participates both ETC and Krebs cycle. It catalyzes the oxidation of succinate to fumarate in the Krebs cycle. During this reaction, succinate provides two electrons, flavin adenine dinucleotide (FAD) forming $FADH_2$. The redox potential of $FAD/FADH_2$ is -220 mV. Then these two electrons travel to Fe-S clusters, while $FADH_2$ is oxidized back to FAD. Two electrons pass through the cytochrome *b* to Q, which is reduced to QH_2. Ubiquinol then transports these two electrons to complex III.

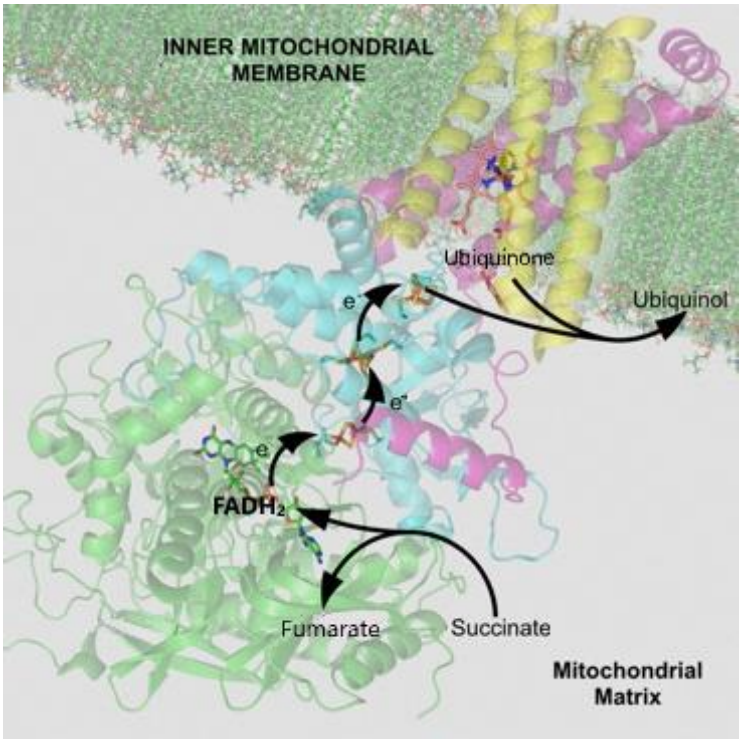

Figure 5 The structure of complex II. [1]

2.2.4.2.1 The production of superoxide anion radical in complex II

In complex II there are two sites of the $O_2^{\bullet-}$ formation, on the FAD and on the Q binding sites. Reduced $FADH_2$ by the succinate from the Krebs cycle can reduce O_2 to H_2O_2 or there

can be created FAD semiquinone (FADH$^{\bullet}$) which can reduce O_2 to $O_2^{\bullet -}$. Alternatively, when Q semiquinone (SQ$^{\bullet}$) is formed, reduction of O_2 by SQ$^{\bullet}$ produces $O_2^{\bullet -}$(Figure 6) (Dröse 2013). The redox potential of Q/ SQ$^{\bullet}$ is 31 mV.

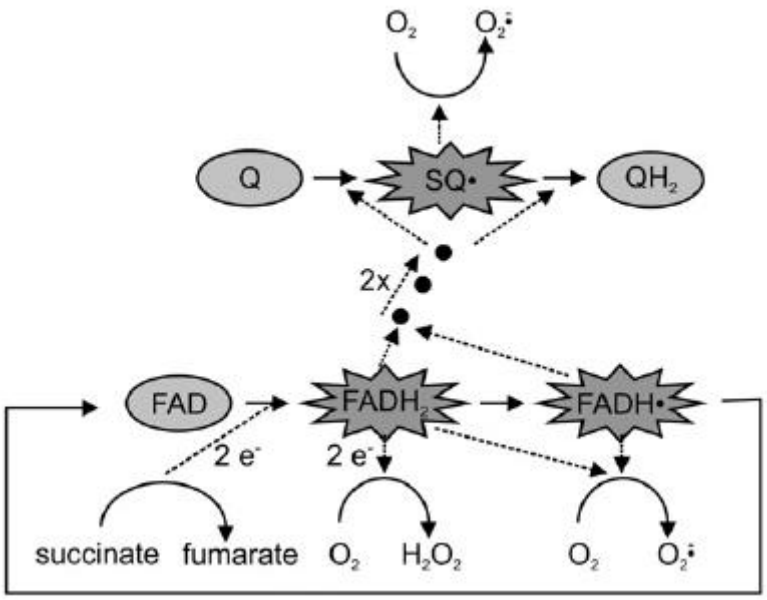

Figure 6 Possibly ways of the superoxide production in complex II. (Dröse 2013)

2.2.4.3 Complex III

Complex III (cytochrome *bc₁* complex) is the third complex in the ETC and plays an important role in the production of ATP. This complex catalyzes a reaction

$$QH_2 + 2\ \text{cytochrom}\ c\ (Fe^{III}) + 2\ H_{in}^{+} \leftrightarrow Q + 2\ \text{cytochrom c}\ (Fe^{II}) + 4\ H_{out}^{+} \quad (14)$$

where cytochrome *c* (Fe^{III}) is reduced to cytochrom *c* (Fe^{II}) by the QH_2, while two protons are transported from the matrix to complex III and four protons are released to the inter membrane space.

Ubiquinol transports two electrons from complex II to complex III. There are two Q-reaction centers in complex III, which are located on the opposite sides of the complex and are mainly formed by the cytochrome *b*. The ubiquinol-oxidation center (Q_0 site) is located closer to the intermembrane space and the ubiquinone-reduction center (Q_i site) is located closer to the matrix (Hunte *et al*. 2003).

The Q-cycle starts at the Q_0 site with the oxidation of QH_2 to Q, while the two electrons go to the high-potential and low-potential chain. The first electron enters the high-potential chain. It is transferred to the binuclear Fe-S cluster of the Rieske protein (Figure 7). Then electron is transferred *via* cytochrome c_1 to cytochrome *c*. Two protons are released to the intermembrane space. The second electron enters the low-potential chain at heme b_L and it is transferred though the heme b_H onto Q on the Q_i site, leading to the formation of $SQ^{\bullet}$ (Ohnishi, Trumpower 1980). The second QH_2 is oxidized on the Q_0 site, then others two protons are released to the intermembrane space and cytochrome *c* is reduced through the high potential chain, meanwhile the second electron is transferred through low potential chain, where reduces $SQ^{\bullet}$ to Q at the Q_i site. This process is ended by the transfer of two protons from matrix to the complex III.

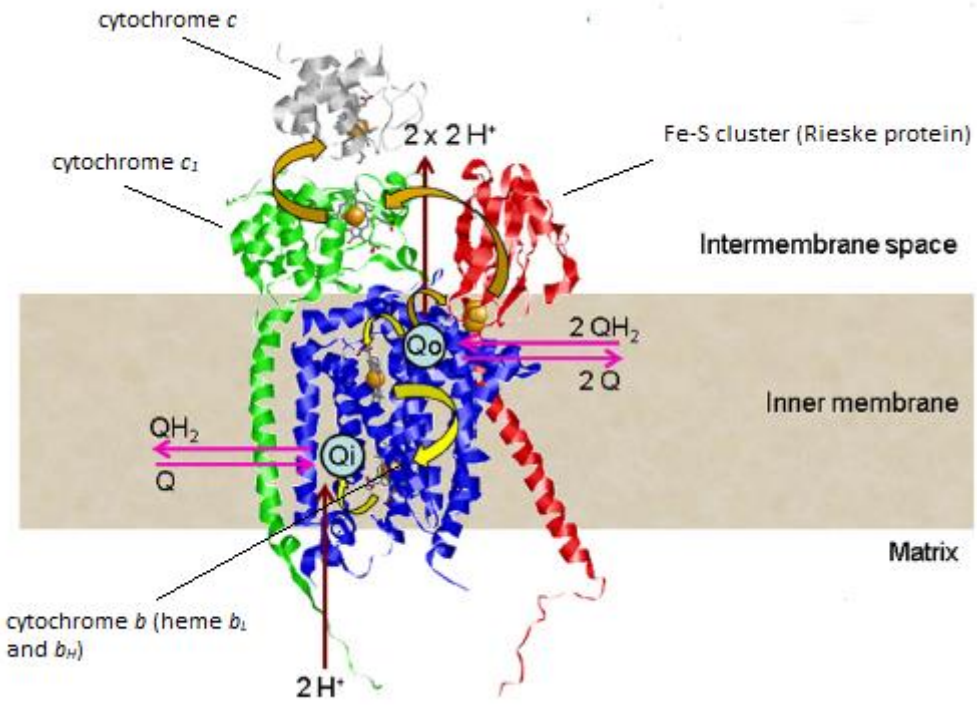

Figure 7 The structure of complex III. (Meunier et al. 2012)

2.2.4.3.1 The production of superoxide anion radical in complex III

The main reductant is $SQ^{\bullet}$ at Q_0 site (Turrens 2003). Ubiquinol is oxidized to Q, thereby protons are released to the intermembrane space and also QH_2 provides an electron to Fe-S cluster, which after that reduces Q to $SQ^{\bullet}$, the redox potential of $Q/SQ^{\bullet}$ is E_m = -36 mV. Then

$SQ^{\bullet}$ can reduce O_2 to $O_2^{\bullet -}$. The production of $O_2^{\bullet -}$ is also possible at Q_i site, where also $SQ^{\bullet}$ reduce O_2 to $O_2^{\bullet -}$.

The most effective inhibitor of complex III is antimycin A. It inhibits oxidation of QH_2 by the inhibition of electrons transport from cytochrome b_H to QH_2.

2.2.4.4 Complex IV

Complex IV (cytochrome *c* oxidase) is the last enzyme of ETC. Each electron from four molecules of cytochrome *c* is transferred to one molecule of oxygen, which is then reduced to two molecules of water and simultaneously four protons are transferred from the matrix to the intermembrane space (15).

$$4\ Fe^{2+}\text{-cytochrom } c + 8\ H^{+}_{in} + O_2 \rightarrow 4\ Fe^{3+}\text{-cytochrome } c + 2\ H_2O + 4\ H^{+}_{out} \qquad (15)$$

The transport of four protons promotes the formation of different proton concentration between the matrix and the intermembrane space. This difference is used to synthase of ATP by ATP synthase.

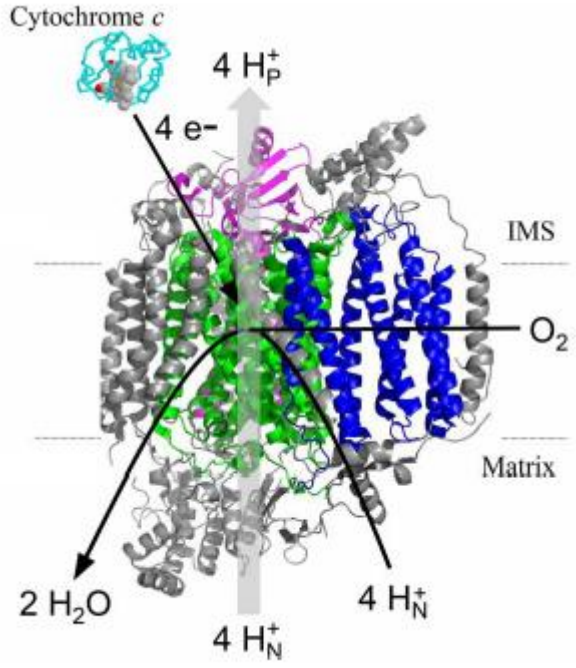

Figure 8 The structure of complex IV. (Rich 2017)

2.3 Carbon-centered radical

Carbon-centered radical ($R^{\bullet}$) is a molecule containing unpaired electron on carbon atom. Biomolecules are oxidized by ROS which leads to formation of $R^{\bullet}$ (11).

$$HO^{\bullet} + RH \rightarrow R^{\bullet} + H_2O \quad (11)$$

In this reaction, hydrogen abstraction from polyunsaturated fatty acid or amino acid causes formation of $R^{\bullet}$.

2.4 Electron paramagnetic resonance spectroscopy

Electron paramagnetic resonance (EPR) spectroscopy is a spectroscopic method that detects species containing unpaired electrons. Electron paramagnetic resonance spectroscopy is an important tool in studies of biological systems that contain paramagnetic species such as substrate radicals, electron-transfer centers and metal ions (Brudvig 1995). This method is based on Zeeman effect. Paramagnetic systems absorb microwave radiation in the presence of an applied magnetic field (Rana *et al*. 2010). This interaction splits the spin energy levels (Figure 9).

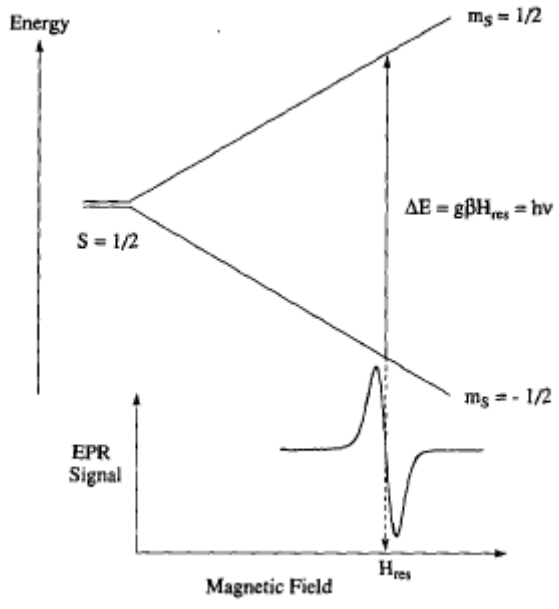

Figure 9 Splitting of the electron spin states by a magnetic field and the EPR signal. (Brudvig 1995)

Due to the very short life-time (ms-ns), we detect radicals by EPR spin-trapping spectroscopy using spin traps. Spin traps create more stable radical adducts, life-time from minutes to hours, which we are able to detect.

3. Aim of the thesis

The aim of this thesis was to detect carbon-centered radical in mitoplasts and submitochondrial particles isolated from the pork heart using electron paramagnetic resonance spectroscopy and spin trap DMPO and subsequently localized its formation.

4. Material and methods

4.1 Chemicals

The major important chemicals used during the work are listed in this paragraph. Spin-trap 5,5-Dimethyl-1-pyrroline *N*-oxide (DMPO) was purchased from (Dojindo laboratories, Japan). Inhibitors rotenone, antimycin A and electron donators NADH and succinate were purchased from Sigma-Aldrich (Germany).

A brief list of other used chemicals are as follows: sodium chloride (NaCl) (Lach-ner, Czech Republic), mannitol (Sigma-Aldrich, Germany), sucrose (Sigma-Aldrich, Germany), EDTA (ethylendiaminetetraacetic acid) (Sigma-Aldrich, Germany), MOPS (3-(N-morpholino) propanesulfonic acid) (Sigma-Aldrich, Germany), SOD (superoxide dismutase) (Sigma-Aldrich, Germany), CAT (catalase) (Sigma-Aldrich, Germany).

4.2 Isolation of submitochondrial particles and mitoplasts

Mitoplasts (MTs) or submitochondrial particles (SMPs) were isolated from pork heart as described by Matsuzaki, Szweda (2007) with a couple modifications. Pork heart was transported from the local slaughterhouse to the laboratory in physiological solution (0,9% NaCl) on ice. The preparation of the heart for centrifugation has been done at 4°C. At first, pork heart was rinsed in ice-cold isolation buffer (210 mM mannitol, 70 mM sucrose, 1 mM EDTA and 10 mM MOPS (pH 7.4)). Then the heart was cut to tiny pieces, frozen in liquid N_2 and pulverized. Tissue was put into 10 mM MOPS, 1 mM EDTA and homogenized. Homogenate was filtered through two layers of nylon bolting cloth. Filtrate was transferred into tubes and centrifuged at 750 g for 5 min at 4°C. The supernatant was centrifuged at 10000 g for 20 min and then the pellet was resuspended in 12 ml of 10 mM MOPS and 1 mM EDTA (pH 7,4). The solution was frozen in liquid N_2, thawed twice and then centrifuged at 10000 g for 20 min. The pellet was resuspended in 6 ml of 10 mM MOPS ans 1 mM EDTA (pH 7.4) and sonicated on ice (8 x 15 s with 30 s intervals). Homogenate was centrifuged at 10000 g for 7 min. The pellet containing unbroken mitoplasts was resuspended in 10 mM MOPS and the supernatant was

centrifuged at 40000 g for 60 min. The pellet containing SMPs was resuspended in 10 mM MOPS. Isolated SMPs were inside-out vesicles (Figure 9).

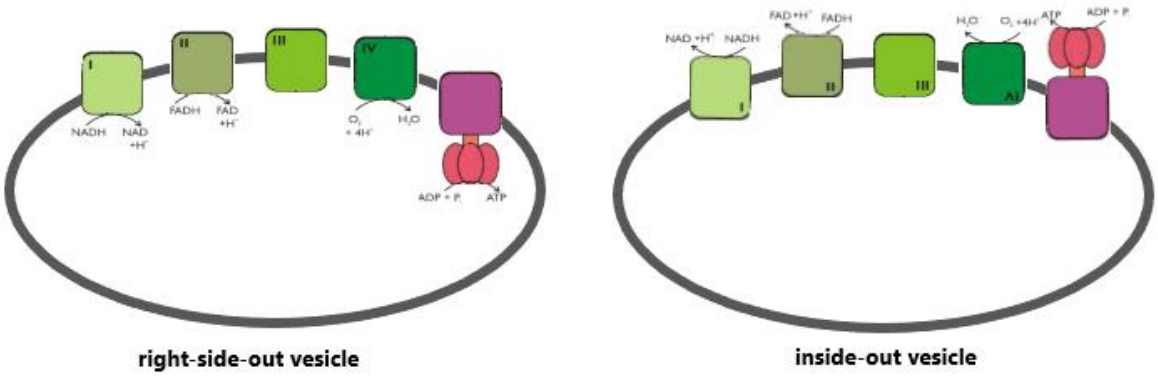

Figure 10 Right-side-out vesicle and inside-out vesicle. (Mitochondrial complexes were borrowed from [2].)

The whole isolation is described on Figure 10. The concentration of mitoplasts and SMPs was measured by Biuret method as describe by Peč *et al*. (2008).

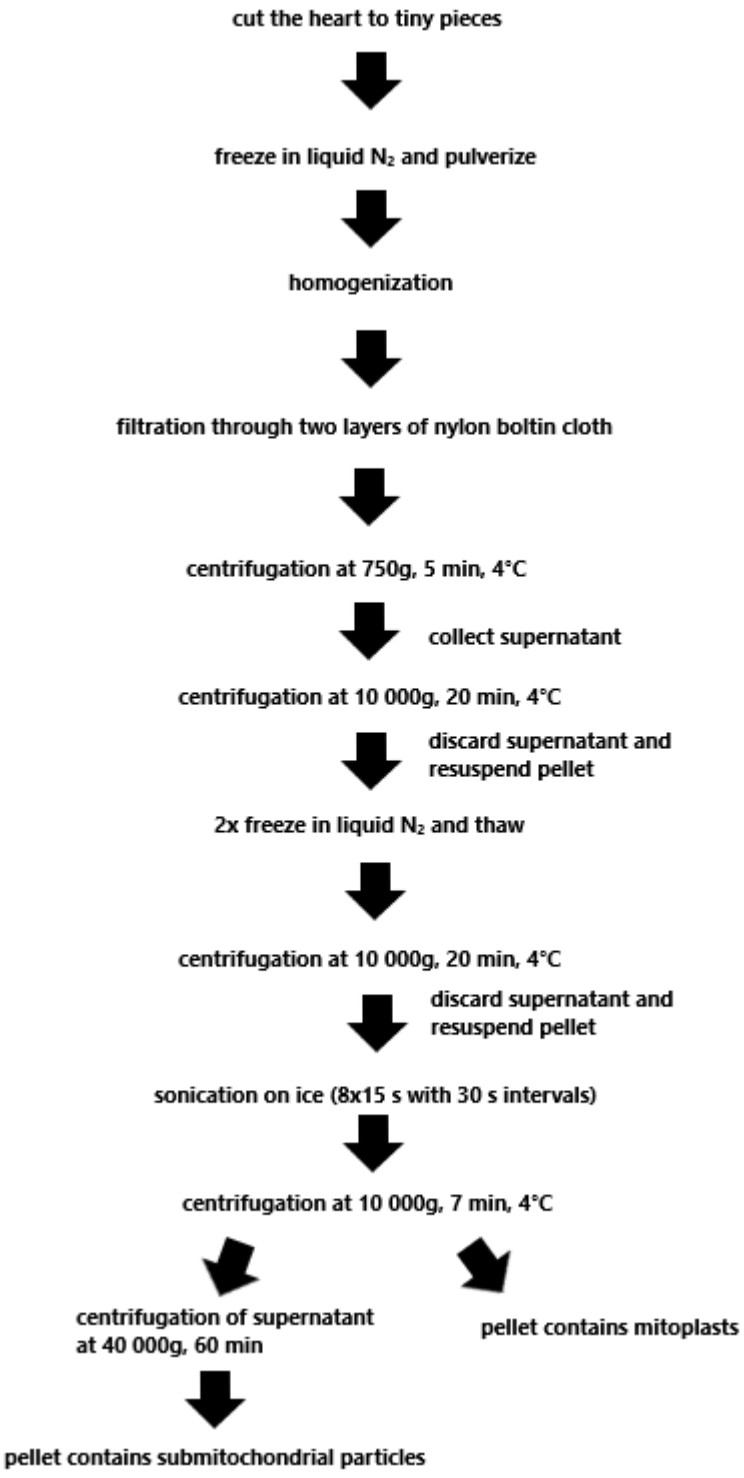

Figure 11 Scheme of isolation of SMPs and MTs.

4.3 Treatment of MTs and SMPs

We examine two different ways of R• production. The succinate-mediated R• production we were examining using electron inhibitor antimycin A and electron donor succinate. Mitoplasts (1mg/ml) and SMPs (1mg/ml) were supplemented with 500 nM antimycin A and 10 mM succinate. The NADH-mediated R• production we were examining using inhibitor antimycin A and rotenone and electron donor NADH. Mitoplasts (1mg/ml) and SMPs (1mg/ml) were supplemented with 500 nM antimycin A or 500 nM rotenone and 10 mM NADH. All the EPR measurements were performed in the presence of chelators to avoid contribution of free metals.

4.4 EPR Spin-Trapping Experiments

EPR spin-trapping spectroscopy was used to measure formation of R• in MPs and SMPs. Detection of R• was accomplished by spin trap 5,5-Dimethyl-1-pyrroline *N*-oxide (DMPO). After treatment, MPs and SMPs were transferred into the glass capillary tube (Blaubrand® intraMARK, Brand, Germany) and DMPO-R adduct EPR spectra were measured in the presence of 50 mM DMPO, 25 mM KCl, 1 mM EDTA, 40 μM DTPA and 10 mM MOPS (pH 7.4). DMPO-R adduct EPR spectra were recorded using an EPR spectrometer MiniScope MS400 (Magnettech, Berlin, Germany). EPR conditions were as follows: microwave power, 10 mW; modulation amplitude, 1 G; modulation frequency, 100 kHz; sweep width, 100 G; scan rate, 1.62 G s^{-1}.

5. Results

Our primary aim was to detect R• using spin trap DMPO in MTs and SMPs. It is well known that reaction of R• and DMPO forms carbon-centered radical adducts (DMPO-R) (Dikalov *et al*. 1996). At first, we examined formation of DMPO-R adduct when electron transport was induced by succinate through complexes II – III – IV in MTs and SMPs (succinate-mediated R• production). Figure 12 shows that no DMPO-R adduct EPR signal was formed even after 30 min incubation. This result indicates that R• is not formed in the succinate-mediated electron transport.

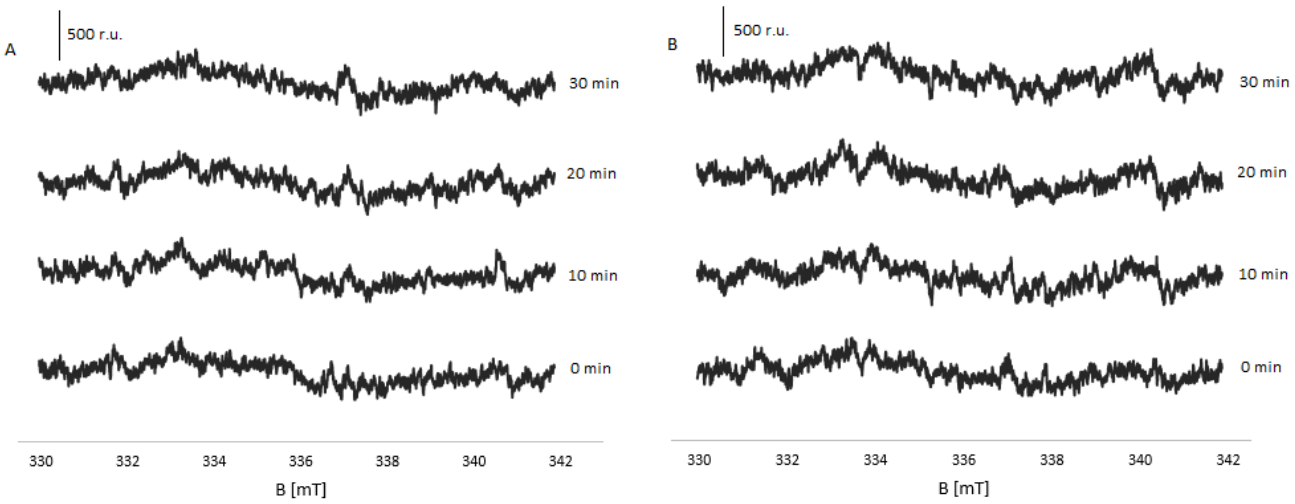

Figure 12 EPR spectra of electron transport through complexes II - III - IV. EPR spectra were recorded immediately after we prepared a mixture of (A) DMPO (50 mM), SMPs (1 mg/ml) supplemented with 500 nM antimycin A and 10 mM succinate, in 10 mM MOPS, 25 mM KCl, 1 mM EDTA and 40 μM DTPA or (B) DMPO (50 mM), mitoplasts (1 mg/ml) supplemented with 500 nM antimycin A and 10 mM succinate, in 10 mM MOPS, 25 mM KCl, 1 mM EDTA and 40 μM DTPA.

Further, we examined R• formation in MTs and SMPs when electron transport through complexes I-III-IV is mediated by NADH (NADH-mediated R• production). Figure 13 shows that DMPO-R adduct EPR signal was observed. DMPO-R adduct generated by SMPs had the strongest signal immediately after a preparation of the mixture and slowly decrease after 10 min of incubation, whereas no recognizable DMPO-R adduct EPR signal was observed after 20 min. In comparison with SMPs, DMPO-R adduct generated by MTs had the strongest signal after 10 min of incubation. After 20 min of incubation there was decreased signal of DMPO-R adduct but still visible; however, after 30 min of incubation there were no observable DMPO-R adduct. This result indicates that R• is formed in the NADH-mediated electron transport.

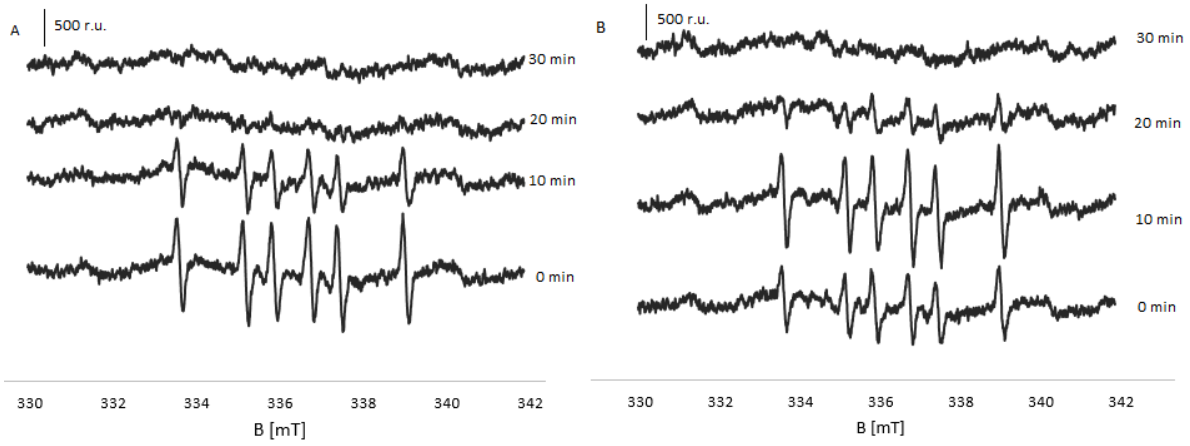

Figure 13 DMPO-R adduct EPR spectra generated by (A) SMPs (1mg/ml) and (B) mitoplasts (1 mg/ml), supplemented with 10 mM NADH, 500 nM antimycin A in 10 mM MOPS, 25 mM KCl, 1 mM EDTA and 40 µM DTPA using spin-trap DMPO (50 mM).

Further, DMPO-R adduct EPR spectra were measured in the presence of inhibitors to determine the origin of R•. At first, we were determined which mitochondrial complex is responsible for R• formation. When antimycin A (an inhibitor of electron transport in complex III) was used, it indicates that complex III is the place of generation R•. When rotenone (an inhibitor of electron transport in complex I) was used, DMPO-R adduct EPR signal was also observed (Figure 14). These results indicate that R• is formed in the complex I.

As we can see in Figure 14, DMPO-R adduct generated in MTs and SMPs using rotenone has smaller signal than when we supplemented MTs and SMPs with antimycin A. This result indicates, that the DMPO-R adduct is produced by RET.

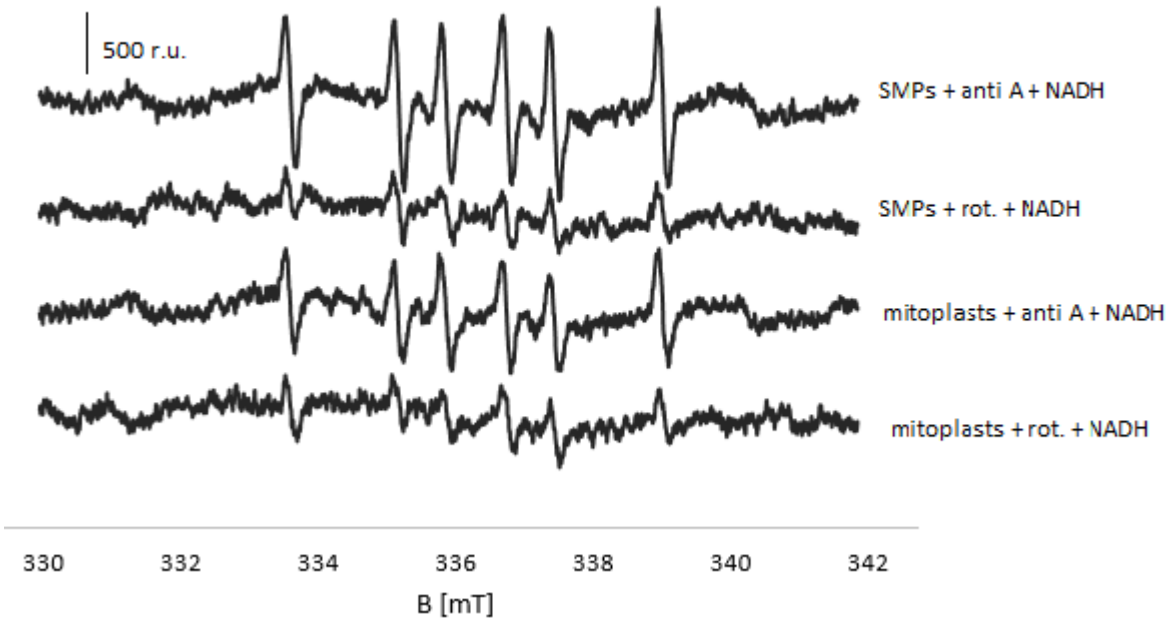

Figure 14 DMPO-R adduct EPR spectra in mitoplasts (1 mg/ml) or SMPs (1 mg/ml) supplemented with 10 mM NADH, 500 nM antimycin A or 500 nM rotenone in 10 mM MOPS, 25 mM KCl, 1 mM EDTA and 40 µM DTPA using spin trap DMPO (50 mM).

Subsequently, we examined whether the DMPO-R adduct is membrane-associated or soluble. For this experiment, we measured the DMPO-R adduct EPR spectra in the supernatant and the pellet of the mixture containing MTs supplemented with NADH and antimycin A. The signal of DMPO-R adduct was detectable only in the supernatant (Figure 15). The signal was decreased due to the redox interaction of the adduct during the preparation. The result indicates that the origin of the DMPO-R adduct is non-membrane protein or a small molecule.

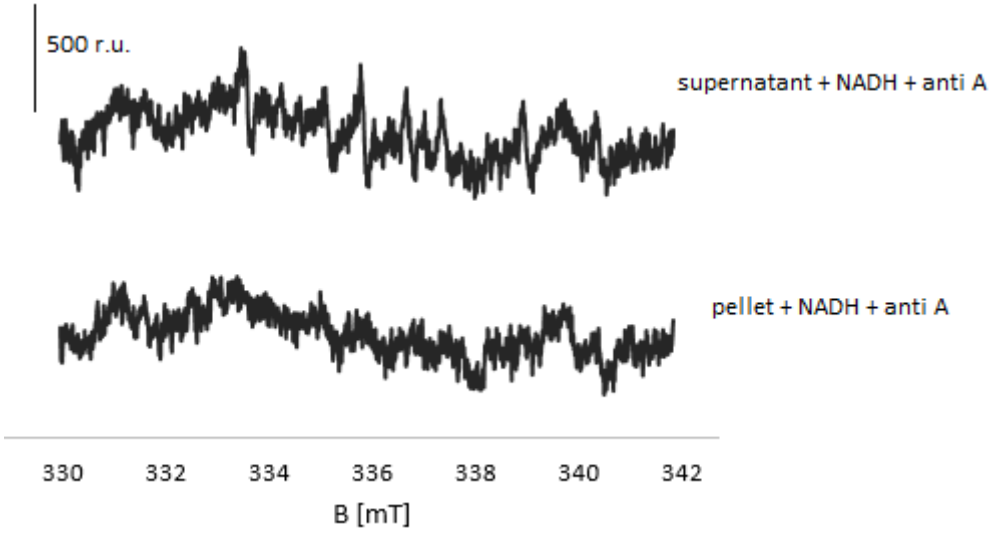

Figure 15 Determination of the origin of carbon-centered radical. Mitoplasts (1mg/ml) were supplemented with 10 mM NADH, 500 nM antimycin A in 10 mM MOPS, 25 mM KCl, 1 mM EDTA and 40 µM DTPA using spin trap DMPO (50 mM). The mixture was centrifuged by low speed centrifugation and then supernatant and pellet was measured.

Next, we measured dependence of DMPO-R adduct on the concentration of NADH. We observed that DMPO-R adduct is NADH concentration dependent (Figure 16).

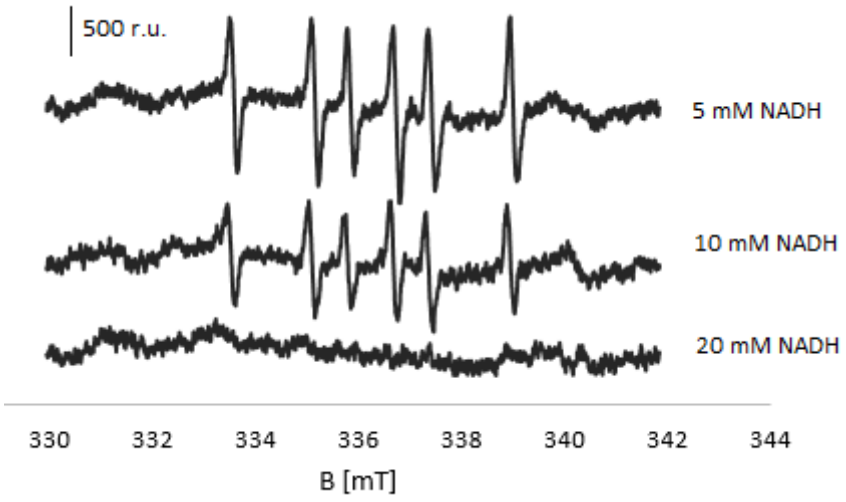

Figure 16 NADH concentration dependency on carbon-centered radical formation. Mitoplasts (1mg/ml) were supplemented with NADH, 500 nM antimycin A in 10 mM MOPS, 25 mM KCl, 1 mM EDTA and 40 µM DTPA using spin trap DMPO (50 mM).

At last we examined SOD and CAT sensitivity. Both SOD and CAT eliminated the DMPO-R adduct in MTs and SMPs (Figure 17).

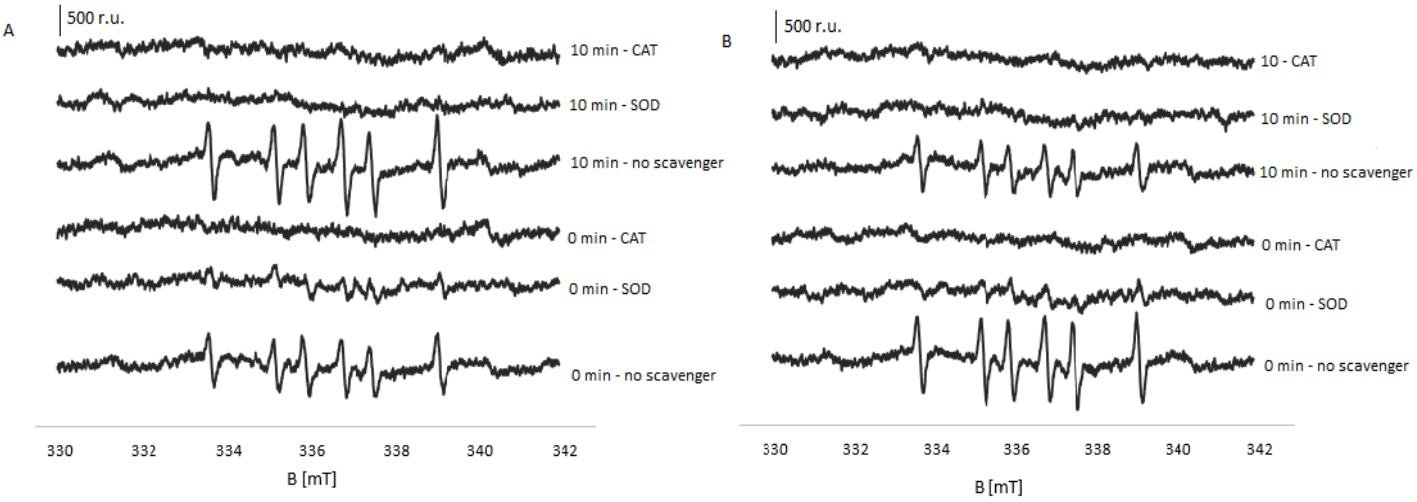

Figure 17 (A) Mitoplasts (1mg/ml) were supplemented with 10 mM NADH, 500 nM antimycin A and SOD (400U/ml) or CAT (400U/ml) in 10 mM MOPS, 25 mM KCl, 1 mM EDTA and 40 µM DTPA using spin trap DMPO (50 mM). (B) SMPs (1mg/ml) wre supplemented with 10 mM NADH, 500 nM antimycin A and SOD (400U/ml) or CAT (400U/ml) in 10 mM MOPS, 25 mM KCl, 1 mM EDTA and 40 µM DTPA using spin trap DMPO (50 mM).

Immediately after we prepared the mixture with SOD, there was a DMPO-R adduct, but after 10 min of incubation there was no signal of DMPO-R adduct. In contrast to SOD, CAT

eliminated DMPO-R adduct immediately after we prepared the mixture and there was no detectable signal.

6. Discussion

In this study, we examined formation by complex III (succinate-mediated R• production) and by complex I (NADH-mediated R• production) in both MTs and SMPs. Our results showed that MTs and SMPs supplied with succinate produced no observable DMPO-R adduct (Figure 12). However, Matsuzaki *et al.* (2011) showed formation of CYPMPO-OOH adduct EPR signal in rat heart SMPs when electron transport was supplied by succinate in the presence of antimycin A. In agreement with this, Han *et al.* (2001) showed that succinate-mediated electron transport in the presence of antimycin A resulted in the formation of DMPO-OH adduct in rat liver MTs. Our results did not show superoxide radical adduct due to the spin trap DMPO. The DMPO-OOH adduct is unstable and its lifetime is about 1 min. The reason why Han *et al.* (2001) detected DMPO-OH and we did not is due to the chelators we used in buffer. However, Han *et al.* (2001) did not use chelators in buffer.

Next, we examined the NADH-mediated R• production. Mitoplasts and SMPs supplied with NADH produced observable DMPO-R adduct. This radical adduct was stable only for 10 mins in both MTs and SMPs (Figure 13) due to the paramagnetic adduct reduction (hydroxylamine adduct) or oxidation (nitrone adduct) to the diamagnetic adduct (Ramirez *et al*. 2007). In Figure 13 we can see that DMPO-R adduct in SMPs has stronger signal than in MTs. This difference is a result of SMPs structure. The structure of SMPs is inside-out vesicle which mean that the matrix side of the inner membrane of mitochondria is oriented to the outside. This orientation allows to NADH easily reach complex I NADH-binding side. There was previous report of detection of carbon-centered radical in MTs. Matsuzaki *et al.* (2011) showed CYPMPO-R radical adduct, DEPMPO-R radical adduct and BMPO-R radical adduct in rat heart MTs using NADH as an electron donor and antimycin A as an inhibitor.

In further, we examined the origin of DMPO-R adduct. We supplemented MTs and SMPs with rotenone instead of antimycin A. In Figure 14 we can see, that MTs and SMPs supplemented with rotenone produced DMPO-R adduct. In comparison to MTs and SMPs supplemented with antimycin A, the signal of DMPO-R adduct produced by MTs and SMPs supplemented with rotenone was decreased. The result indicates, that carbon-centered radical is produced in complex I by RET. Scialò *et al.* 2017 show that rotenone decreased amount of ROS produced by RET (Figure 4).

Subsequently we examined if the carbon-centered was membrane associated or soluble in origin. For this experiment we centrifugated mixture of MTs, NADH, antimycin A and DMPO for 5 mins. Results revealed that all the carbon-centered radical was in supernatant (Figure 15). The EPR spectra of DMPO-R adduct was decreased due to the redox interaction of the adduct during the preparation. Matsuzaki *et al.* (2011) revealed that all the CYPMPO-R radical adduct was in supernatant and they suggested that the observed carbon-centered radical is non-membrane protein or a small molecule.

Next, we measured NADH concentration dependence. We observed, that DMPO-R adduct is NADH concentration dependent (Figure 16). With increasing concentration of NADH, signal of DMPO-R was decreased. The rate of $O_2^{\bullet -}$ production in complex I is influenced by NADH concentration due to the bound nucleotides which block access to the $FMNH_2$ and then O_2 cannot be reduced to $O_2^{\bullet -}$. Thus, reduction of O_2 is inhibited as the NADH concentration increases (Birrell *et al.* 2009).

At last, we examined the SOD and CAT sensitivity. The DMPO-R adduct was SOD and CAT sensitive in both MTs and SMPs (Figure 17). Molecular oxygen is reduced by $FMNH_2$ to $O_2^{\bullet -}$, which is dismutated by SOD to H_2O_2. Hydrogen peroxide produce $HO^{\bullet}$ by one-electron reduction catalyzed by bounded metal. Then $HO^{\bullet}$ is eliminated by CAT.

7. Conclusion

The aim of this bachelor thesis was to detect R• in heart mitochondria using EPR spectroscopy. We detected DMPO-R adduct formed in NADH-mediated electron transport in MTs and SMPs. This DMPO-R adduct was stable only for 10 min due the to transformation of paramagnetic adduct to diamagnetic adduct. We found out that R• is not formed in the succinate-mediated electron transport.

We observed that R• is formed in complex I during the RET due to the decrease of DMPO-R adduct using rotenone as an inhibitor instead of antimycin A. The separation of lipid soluble and water-soluble compounds showed is R• formed in polar phase. We also authenticated that the increase of NADH concentration inhibits the formation of R• due to blocking access to $FMNH_2$.

We suggest that the whole process of R• formation is following: at first two electrons from NADH are donated to complex I, they are transferred to Q and reduced it to QH_2, electrons from the over-reduced Q-pool are transferred back to FMN in complex I, this FMN is reduced to $FMNH_2$, $FMNH_2$ reduced O_2 to $O_2^{\bullet -}$, $O_2^{\bullet -}$ is reduced to H_2O_2, H_2O_2 is reduced to HO• by bonded metals and finally HO• reduced NADH to our detected R• (Figure 18).

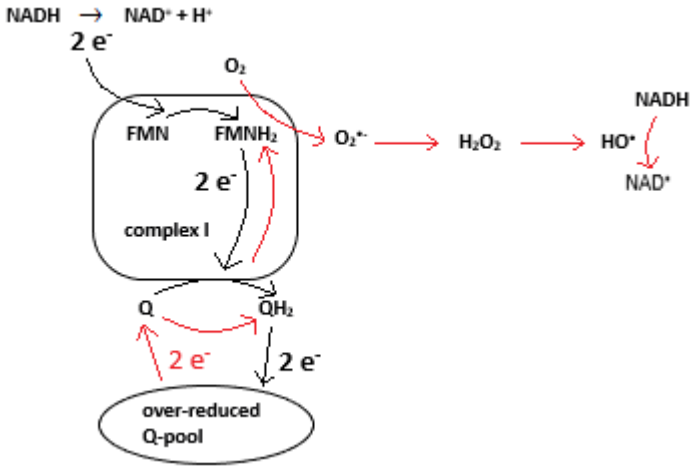

Figure 18 Scheme of NAD• formation.

The carbon atom with unpaired electron is one of the double bonded carbon atoms in NADH (Figure 19).

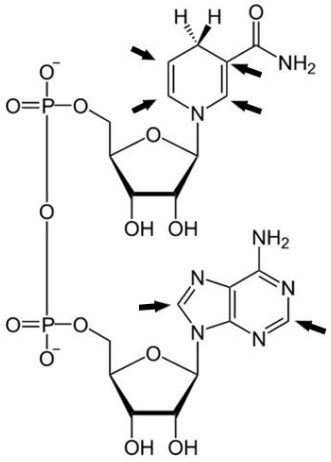

Figure 19 Structure of NADH with highlighted double bonded carbon atoms. (Structure of NADH was borrowed from [3].)

In the future, there are still interesting ways to investigate in mitochondria. For example, the succinate-mediated electron transport or the FET. This bachelor thesis is focused only on the detection of R•. Next studies could be focused on the detection of ROS in mitochondria.

8. References

Alberts B., Johnson A., Lewis J., Raff M., Roberts K., Walter P. (2002) *Molecular biology of the cell.* 4th ed, Garland science, New York, 1616 stran.

Birrell J.A, Yakovlev G., Hirst J. (2009) reaction of the flavin mononucleotide in complex I: a combined mechanism describes NADH oxidation coupled to the reduction of APAD+, ferricyanide, or molecular oxygen. *Biochemistry* **48**, 12005-12013.

Blake D.R., Allen R.E., Lunec J. (1987) Free radicals in biological systems. *British medical bulletin* **43**, 371-385.

Brand M.D. (2010) he sites and topology of mitochondrial superoxide production. *Experimental gerontology* **45**, 466-472.

Brandt U. (2006) Energy converting NADH:quinone oxidoreductase (complex I). *Annual rewiew of biochemistry* **75**, 69-92.

Brudvig G.W. (1995) Electron paramagnetic resonance spectroscopy. *Methods in enzymology* **246**, 536-554.

Dikalov S., Kirilyuk I., Grigor'ev I. (1996) Spin trapping of O-, C-, and S-centered radicals and peroxynitrite by 2H-imidazole-1-oxides. *Biochemical and biophysical research communications* **212**, 616-622.

Devasagayam T., Tilak J.C., Boloor K.K., Sane K.S., Ghaskadbi S.S., Lele R.D. (2004) Free radicals and antioxidants in human health: current status and future prospects. *Journal of association of physicians of India* **52**, 794-804.

Dröse S. (2013) Differential effects of complex II on mitochondrial ROS production and their relation to cardioprotective pre- and postconditioning. *Biochimica et biophysica acta* **5**, 578-587.

Halliwell B., Gutteridge J.M.C. (2015) *Free radicals in biology & and medicine.* 5th ed., Oxford university press, Oxford, 823 stran.

Han D., Williams E., Cadenas E. (2001) Mitochondrial respiratory chain-dependent generation of superoxide anion and its release into the intermembrane space. *Biochemical journal* **353**, 411-416.

Hansford R.G., Hogue B.A., Mildaziene V. (1997) Dependence of H_2O_2 formation by rat heart mitochondria on substrate availability and donor age. *Journal of bioenergetics and biomembranes* **29**, 89-95.

Hirst J. (2013) Mitochondrial complex I. *Annual review of biochemistry* **82**, 551-575.

Hirst J., Roessler M.M. (2016) Energy conversion, redox catalysis and generation of reactive oxygen species by respirátory complex I. *Biochimica et biophysica acta* **7**, 872-883.

Hunte C., Palsdottir H., Trumpower B.I. (2003) Protomotive pathways and mechanism in the cytochrome bc_1 complex. *FEBS letters* **545**, 39-46.

Chipuk J.E., Bouchier-Hayes L., Green D.R. (2006) Mitochondrial outer membrane permeabilization during apoptosis: the innocent bystander scenario. *Cell death & differentiation* **13,** 1396-402.

Kussmaul L., Hirst J. (2006) The mechanism of superoxide production by NADH:ubiquinone oxidoreductase (complex I) from bovine heart mitochondria. *Proceedings of the national academy of science* **103**, 7607-7612.

Löffler G., Petrides P.E. (1988) *Physiologische chemie.* 4th ed., Springer, Berlin, 288 stran.

Matsuzaki S., Szweda L.I. (2007) Inhibition of Complex I by Ca^{2+} reduces electron transport activity and the rate of superoxide anion production in cardiac submitochondrial particles. *Biochemistry* **46**, 1350-1357.

Matsuzaki S., Kotake Y., Humphries K.M. (2011) Identification of mitochondrial electron transport chain-mediated NADH radical formation by EPR spin-trapping techniques. *Biochemistry* **50**, 10792-10803.

Moss G.P., Smith P.A.S., Tavernier D. (1995) Glossary of class names of organic compounds and reactive intermediates on structure. *Pure and applied chemistry* **67**, 1307-1375.

Meunier B., Fisher N., Ransac S., Mazat J.-P., Brasseur G. (2012) Respiratory complex III dysfunction in humans and the use of yeast as a model organism to study mitochondrial myopathy and associated diseases. *Biochimica et biophysica acta* **11-12**, 1346-1361.

Ohnishi T., Trumpower B.I. (1980) Differential effects of antimycin on ubisemiquinone bound in different enviroments in isolated succinate:cytochrome *c* reductase complex. *The journal of biological chemistry* **255**, 3278-3284.

Peč P., Šebela M., Luhová L., Frébort I., Zajoncová L., Petřivalský M. (2008) *Laboratorní cvičení z biochemie.* 3rd ed., Univerzita Palackého v Olomouci, Olomouc, Česká republika, 99 stran

Pham-Huy L.A., He H., Pham-Huy Ch. (2008) Free radicals, antioxidants in disease and health. *Internationl journal of biomedical science* **4**, 89-96.

Ramirez D.C., Gomez-Mejiba S.E., Manson R.P. (2007) Immuno-spin trapping analyses of DNA radicals. *Nature protocols* **2**, 512-522.

Rana S., Chawla R., Kumar R., Singh A., Zheleva A., Dimitrova Y., Gadjeva V., Arora R., Sultana S., Sharma R.K. (2010) Electron paramagnetic resonance spectroscopy in radiation research: Current status and perspectives. *Journal of pharmacy and bioallied sciences* **2**, 80-87.

Rich P.R. (2017) Mitochondrial cytochrome *c* oxidase: catalysis, coupling and controversies. *Biochemical society transaction* **45**, 813-829.

Sazanov L. A. (2015) A giant molecular proton pump: structure and mechanism of respiratory complex I. *Nature reviews molecular cell biology* **16**, 375-388.

Scialò F., Fernández-Ayala D.J., Sanz A. (2017) Role of mitochondrial reverse electron transport in ROS signaling: potential roles in health and disease. *Frontiers in physiology* **8**: 428.

Takeshige K., Minakami S. (1979) NADH- and NADPH-dependent formation of superoxide anions by bovine heart submitochondrial particles and NADH-ubiquinone reductase preparation. *Biochemical journal* **180**, 129-135.

Turrens J. F. (2003) Mitochondrial formation of reactive oxygen species. *The journal of physiology* **552**, 335-344.

Voet D.J., Voet J.G., Pratt Ch.W. (2012) *Principles of biochemistry*. 4th ed, Wiley, UK, 1208 pages.

Waris G., Ahsan H. (2006) Reactive oxygen species: Role in the development of cancer and various chronic conditions. *Journal of carcinogenesis* **5**: 14.

9. Internet sources of figures

[1] Complex II. In: Kinetoplastid Metabolism [online]. Institute de Duve, Université catholique de Louvain, B-1200 Brussels, Belgium [cit. 2018-05-07]. Dostupné z: http://big.icp.ucl.ac.be/~opperd/metabolism/Kinetoplastida/Blog/Entries/2016/2/1_Complex_II_or_succinate_dehydrogenase.html

[2] Electron transport chain. In: Astarbiology [online]. [cit. 2018-05-07]. Dostupné z: http://astarbiology.com/aqa/electron-transport-chain/

[3] NADH. In: Anatomy&Physiology [online]. [cit. 2018-05-07]. Dostupné z: https://content.byui.edu/file/a236934c-3c60-4fe9-90aa-d343b3e3a640/1/module8/readings/atp_nad_fad.html

Errata

6. 6. 2018

s. 1 In biological systems, reactive oxygen species are formed in metabolic processes and play an important role in the cell signalling and homeostasis.

s. 3 “protonated form “

s. 4 “with no unpaired electron”

s. 4 “a strong oxidant known to oxidize biomolecules”

s. 7 “Complex I catalyses”

s. 7 “Complex I receives NADH”

s. 9 “It is well known that rotenone knows”

s. 9 “$FMNH_2$ which produces $O_2^{\bullet -}$“

s. 11 “Possible ways”

s. 13 “is used to synthase ATP”

s. 17 “with a couple of modifications”

s. 18 “as described by”

s. 22 “we determined which mitochondrial complex is responsible”

s. 24 popisek Fig. 17 “were supplemented”

s. 27 “Hydrogen peroxide produces”