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Effect of oxygen levels in cellular activity

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Abstract

Energy for life is produced inside the internal human energy factory, the mitochondrion, where low oxygen levels are likely to be correlated with stressful behaviors. These include thriving for performance, competition and perfection. One way to build a sustainable society is to reduce the daily stress, allowing human energy to target the control of energy resources and environmental safeguarding. So, it is important to assess the impact of decreased oxygen (hypoxia) in the body, especially in the brain, as a small imbalance in brain activity can affect vital energy production essential for sustainable life activities.

This work addressed fluorescence changes associated with the formation of FAD and of reactive oxygen species (ROS), evoked by different levels of hypoxia. Exposing the slices to 0% and 21% O_2, caused an increase in FAD autofluorescence. Upon reoxygenation (95% O_2) the 0% O_2 trace did not recover, while the 21% O_2 signal decreased reaching the baseline. In contrast, at 40% and 65% O_2 the FAD fluorescence decreased reversibly. Regarding ROS fluorescence, no significant changes were observed during the application of 0% and 21% O_2. However, at 40% and 65% O_2, the signals were enhanced recovering partially when the initial oxygen level (95% O_2) was reintroduced.

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Keywords: Flavin adenine dinucleotide (FAD); H_2DCFDA fluorescent indicator; Hippocampal slices; Hypoxia; Reactive oxygen species (ROS); Stress

1. Introduction

Living demands energy production in the body within sustainable constraints regarding oxygen availability for cellular electron transfer chain mechanisms in mitochondria. This is likely associated with hypoxia events evoked by stress. The Institute for Sustainable Communities (ISC), in 1995, defined essential elements for a sustainable

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community: leadership, civic engagement and responsibility, ecological integrity, economic security and social well-being [1]. But the stress, concerns and anxiety that the majority of society often face in the workplace, school and family environments are one of the greatest threats to building strong and sustainable communities. Due to record levels of stress and anxiety around the world, many people are not living their lives in a healthy way, since the respiratory activity becomes ineffective and very superficial. As a consequence, the production of mitochondrial energy may be compromised. There is evidence that the lifestyle of people in the "blue zones" contributes to longevity, being low stress levels one of the main reasons [2].

Breathing is essential and has beneficial effects on health, well-being, stress reduction, and energy production. But in the most critical situations of anxiety or stress, loss of breath control is inevitable [3]. Low levels of oxygen in the tissues, called hypoxia, are associated with several diseases such as cancer, diabetes, heart and degenerative diseases [4].

Recently, the work that won the Nobel Prize for Medicine and Physiology in 2019, reveals the molecular mechanisms that explain physiological adaptations in situations of hypoxia. Most genes activated during hypoxia are regulated by the hypoxia inducible factor (HIF) [5,6]. This factor binds to fragments of DNA, activating the transcription of more than two hundred genes that allow cells to adapt to the hypoxia environment [7,8]. But the ability to adapt during deprivation of normal concentrations of oxygen, does not guarantee that human health remains unharmed in the long term. The brain is the most susceptible organ to hypoxia, since it is a major consumer of energy [9]. Recent studies report damage causing special memory and learning deficits and increased oxidative stress, evoked by hypoxia, in various areas of the brain, such as the hippocampus, which is involved in cognition and memory processes [10].

In the cellular respiratory process, HIF directly affects mitochondrial function, as it modulates components involved in the electron transport chain (ETC) inducing major changes in structure, function and dynamics, namely in the production of energy in the form of ATP [8,11,12]. In addition, HIF also promotes anaerobic glucose metabolism, which is necessary to produce energy when low oxygen does not support oxidative phosphorylation in mitochondria [3,4,13]. When the oxygen supply is not enough, ETC's activity is decreased and reactive oxygen species (ROS), such as superoxide (O_2^-) and hydrogen peroxide (H_2O_2), are produced in excess [14,15]. The increase in ROS leads to oxidative stress that is associated with the development of CNS diseases involving cognitive function and memory processes [10]. HIF is degraded under normoxic conditions, existing evidence that ROS produced in mitochondria are responsible for HIF stabilization [16].

Mitochondrial function and oxygen metabolism are strongly involved in the progression of brain disorders, including Parkinson's and Alzheimer's disease and schizophrenia [17]. The number of people with these diseases is rapidly increasing [18], being necessary to stop this growth in order to move towards a sustainable society. For this reason, it is essential to understand the mitochondrial changes induced by different concentrations of oxygen. The aims of the present study, performed in rat hippocampal slices, were to study autofluorescence and ROS associated fluorescence changes, under hypoxic conditions (0%; 21%; 40% and 65% of oxygen, for 30 min). The first type of traces originated from the intracellular coenzyme flavin adenine dinucleotide (FAD), which is one of the main electron donors involved in the production of energy in the ETC. The ROS signals were obtained, after correction for the autofluorescence, from the traces recorded from slices incubated with the fluorescent ROS indicator H_2DCFDA.

2. Materials and methods

2.1. Hippocampal slices preparation

The fluorescence studies were performed in hippocampal slices from female Wistar rats (8 to 13 weeks, some with 16 to 18 days of gestation). Females were anesthetized with isoflurane, and after cervical dislocation were sacrificed by decapitation. The brain was rapidly removed and the hippocampus was isolated to make slices (400 μm thick) using a set of parallel blades. The hippocampal slices were placed in an oxygenated (95% O_2/ 5% CO_2), solution of extracellular artificial cerebrospinal fluid (ACSF), at room temperature. This solution was prepared with ultrapure water (18.2 MΩ cm) (Interlab Direct — Pure water system) and had the following composition (in mM): 124.0 NaCl, 3.5 KCl, 24.0 $NaHCO_3$, 1.25 NaH_2PO_4, 2.0 $CaCl_2$, 2.0 $MgCl_2$, 10.0 D-glucose.

2.2. Optical measurements

After an hour at rest some slices were incubated with the ROS indicator H_2DCFDA (10 nM), for 1h. These slices allowed the detection of total fluorescence, i.e., the sum of the FAD autofluorescence, recorded from unincubated slices, with the ROS associated fluorescence. For the measurements the slices were transferred to the perfusion chamber of a non-inverted microscope (Zeiss Axioskop), with a perfusion rate of 1.5–2 mL/min determined by a peristaltic pump (GILSON-MINI PLUS-3). The microscope was equipped with a silicon photodiode (Hamamatsu-S1226-Series, 1 mm^2) and a xenon lamp (XBO 75 W/2). The light was focused to the CA3 mossy fiber region of the slice, by means of a water immersion lens (40x N.A. 0.75; 1.6 mm working distance) and collected via a similar lens.

In the present work, the autofluorescence changes had origin in the reduced/oxidized state transitions of the intrinsic protein $FADH_2$ / FAD. A 480 ± 10 nm excitation filter and a 500 nm high-pass emission filter were used. In all experiments the protocol was the following: 10 min of normoxia (95% O_2/ 5% CO_2), 30 min of hypoxia and 30 min of reoxygenation (normoxia). The gas mixtures used to simulate the hypoxic conditions were: 0% O_2/ 95% N_2/ 5% CO_2; 21% O_2/ 74% N_2/ 5% CO_2; 40% O_2/ 55% N_2/ 5% CO_2; 65% O_2/ 30% N_2/ 5% CO_2.

Fluorescence signals were converted using a 16-bit I/V converter and processed using the National Instruments Signal Express 2013 software. Data were recorded at 0.6 s intervals, being the sets of consecutive 100 points replaced by their average, i.e., by points that were plotted at 1 min intervals. The normalized fluorescence signals from the incubated slices were corrected for autofluorescence subtracting, point by point, the corresponding FAD signals. Traces were normalized calculating the ratio between each fluorescence point and the average of the first 10 points, which form the baseline. Data are reported as mean ± S.E.M. Statistical significance was evaluated using the Mann–Whitney U-test ($p < 0.05$).

The drugs used were NaCl (José Manuel Gomes dos Santos, Lda, Odivelas, Portugal) (purity ≥ 99.7%), $MgCl_2$ (Merck, Lisbon, Portugal), $NaHCO_3$ (purity ≥ 99.7%), KCl (purity ≥ 99.0%), NaH_2PO_4 (purity ≥ 99.0%), $CaCl_2$ (purity ≥ 99.0%) and D- Glucose (purity ≥ 99.5%) (Sigma-Aldrich, Sintra, Portugal). H_2DCFDA (Invitrogen by Thermo Fisher Scientific, USA).

3. Results and discussion

Hippocampal slices were exposed to various hypoxic conditions, for 30 min, as shown in Fig. 1. The results in Fig. 1a reveal the different profiles associated with FAD fluorescence. When the hippocampal slices were exposed to the more intense hypoxic situations, 0% and 21% O_2, the FAD signals increased, by 14.0 ± 4.0% and 6.1 ± 1.1%, at 35–40 min (n = 3), respectively. During the reoxygenation period, the changes in FAD fluorescence were irreversible for 0% O_2 and reversible in the cases with 21% O_2.

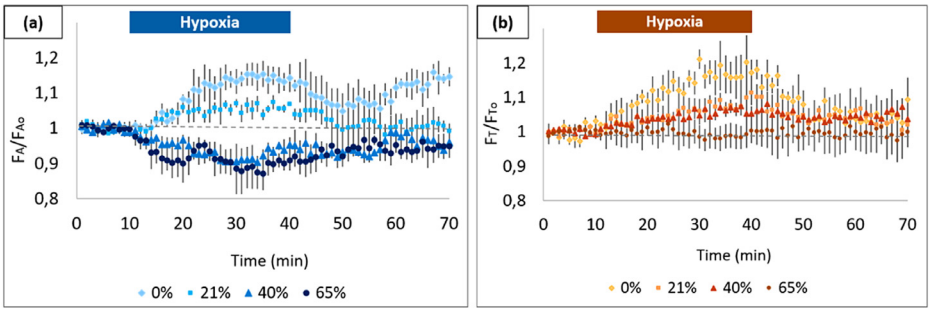

Fig. 1. Fluorescence signals obtained in normoxic (95% O_2, 0 to 10 min), hypoxic (0%–65% O_2, 10 to 40 min) and normoxic (95% O_2, 40 to 70 min) conditions. (a) Changes in FAD autofluorescence (F_A) (n = 3). (b) Total fluorescence signals (F_T) from slices incubated with the ROS indicator H_2DCFDA (n = 3). The points represent the mean ± S.E.M.

On the other hand, under less hypoxic conditions, 40% and 65% O_2, the intensity of the signals decreased by 5.7 ± 1.9% and 9.6 ± 2.5%, at 35–40 min (n = 3), respectively. This change can compromise energy production. During reoxygenation (40–70 min) the signals increased slowly towards the baseline values, without reaching it, suggesting that the changes in question are only partially reversible.

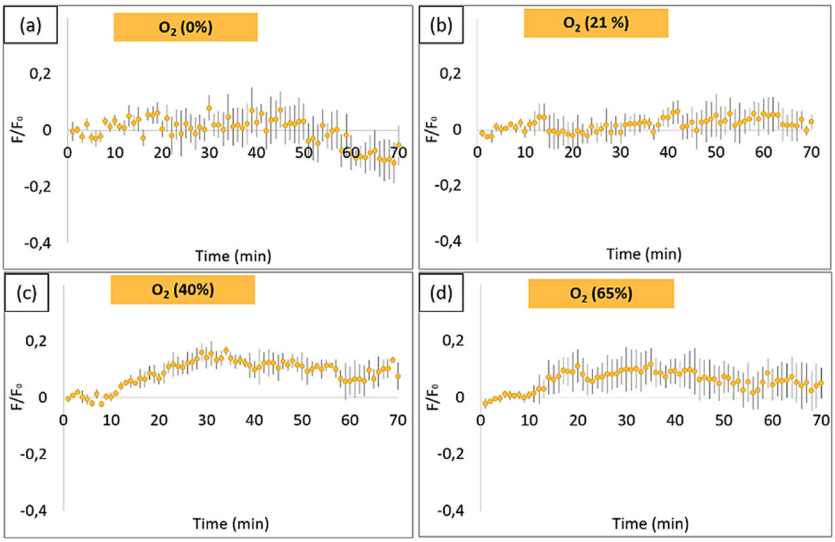

Fig. 2. ROS signals obtained with the indicator H_2DCFDA, corrected by the FAD autofluorescence. (a) 0% O_2 (n = 3). (b) 21% O_2 (n = 3). (c) 40% O_2 (n = 3). (d) 65% O_2 (n = 3). F – ROS fluorescence; F_0 - Baseline ROS fluorescence.

The imbalance between the production and the elimination of ROS is an important indicator of mitochondrial oxidative stress. To investigate this ROS imbalance, some slices were incubated with the H_2DCFDA indicator that makes fluorescent complexes with ROS. Fig. 1b shows the total fluorescence traces, which correspond to the combined FAD and ROS fluorescence. When the slices were exposed to the hypoxic mixtures of 0%, 21% and 40% O_2 (10–40 min) there was a partially reversible increase in total fluorescence. On the other hand, at 65% O_2 no significant changes were observed. Removing the contribution of FAD fluorescence (Fig. 1a) from the total fluorescence records (Fig. 1b), gives the true ROS fluorescence signals, which can be observed in Fig. 2.

The slices exposed to 0% O_2 (Fig. 2a) do not show significant changes in ROS fluorescence. This result is in accordance with the studies by Clanton (2007) and Schumaker (2002), which state that during severe hypoxic conditions (95% N_2 and 5% CO_2), the absence of O_2 limits the production of mitochondrial ROS due to the lack of O_2 as a substrate for electrons. This form of ROS is essential to trigger HIF stabilization, but under the mentioned conditions it is not generated [7,19]. In the 21% O_2 experiment (Fig. 2b), there are also no clear ROS changes.

On the other hand, the application of 40% O_2 (Fig. 2c) caused a 12.0 ± 2.7% increase in ROS fluorescence, at 35–40 min (n = 3). In the case of 65% O_2(Fig. 2d), there was an enhancement of 8.7 ± 4.5%, at 35–40 min (n = 3). This increase can cause changes in energy production in the form of ATP. However, in both cases, during the reoxygenation period (40–70 min), the ROS signal recovered only slightly.

The results in Fig. 1a suggest that the hypoxic states induced by 0% and 21% O_2 initiated a cellular adaptive response that can be mediated by HIF. Fuhrmann and Brüne (2017) [11], Papandreou et al. (2006) [13], Semenza et al. (1991) [6] reported that activation of HIF decreases the activity of the TCA (tricarboxylic acid) cycle by inducing PDK (pyruvate dehydrogenase kinase) which inhibits the activity of the enzyme pyruvate dehydrogenase. As a consequence, the reduction of FAD to $FADH_2$ (Fig. 3) decreases in agreement with the results of Fig. 1a, that show an enhancement in FAD fluorescence under severe hypoxia (0% and 21% O_2).

On the contrary, in the he experiments with 40% and 65% O_2, the same intrinsic fluorescence was reduced. This behavior may be a consequence of the decrease in ETC activity, since 90% of oxygen that reaches the mitochondria is consumed by the IV complex (Fig. 3). A smaller activity of this complex decreases the transfer of electrons throughout the ETC and, consequently, the activity of the other complexes is altered [20]. Thomas and Ashcroft (2019) found that in the CA1 area of hippocampal slices, a 50% decrease in O_2 caused a reduction in complex I activity, which translated into a prolonged increase in NADH [21]. Sasaki et al. (2018) simulated hypoxic situations (95% N_2, 5% CO_2) in slices of cerebral cortex, for 15 min and concluded that the oxidation of NADH was inhibited [22].

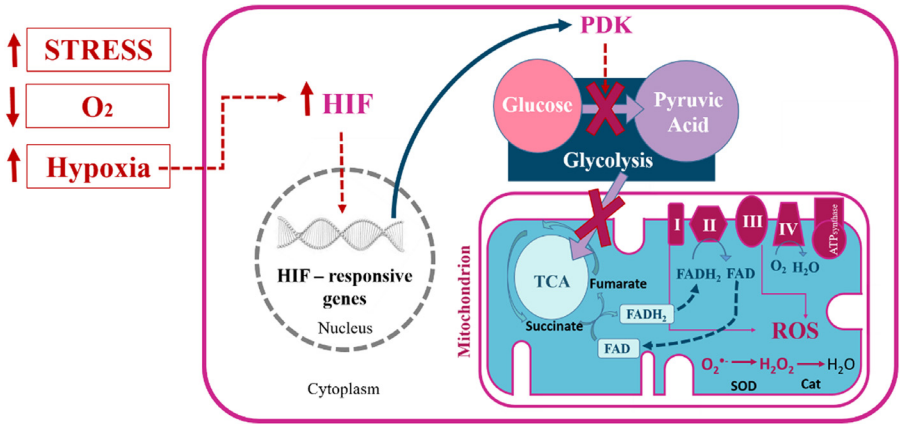

Fig. 3. Mechanism by which HIF triggers the shift to anaerobic glucose metabolism. The activity of the TCA cycle is decreased by inducing PDK which inhibits the activity of pyruvate dehydrogenase. HIF — hypoxia-inducible factor. PDK — pyruvate dehydrogenase kinase. TCA — tricarboxylic acid cycle. ROS — reactive oxygen species.

According to Thomas and Ashcroft (2019) the decrease in ETC activity causes an increase in the probability of unwanted transfer of electrons to oxygen, leading to arise in ROS production [22]. This idea is in line with the increase in ROS observed with 40% and 65% O_2 (Fig. 2c, d). In addition, Coimbra-Costa et al. (2017) [16] and Lages et al. (2015) [17] state that, under hypoxic conditions, antioxidant defense systems, such as superoxide dismutase (SOD) and glutathione peroxidase (GPx), significantly decrease in the brain. The consequent ROS overload is a major concern, since these species are highly reactive and capable of oxidizing lipids, proteins and DNA, leading to structural and functional cellular changes that can cause oxidative damage, apoptosis, necrosis in neurons and decreased energy production.

4. Conclusions

Energy is the utmost factor supporting life that cannot exist without proper cellular oxygenation. Therefore, oxygen is vitally important for life and specially for the brain, which coordinates and controls body functions. Brain tissue is particularly susceptible to changes in oxygen supply and this work supports the idea that short periods of hypoxia can alter the cellular redox state that leads to oxidative stress and neuronal damage.

The results suggest that strong hypoxic situations (0% and 21% O_2) modify the mitochondrial tricarboxylic acid cycle, resulting in an increase in FAD fluorescence. It is also considered that due to the lack of oxygen, there were no significant changes in ROS fluorescence. On the other hand, it is possible that during milder hypoxia states (40% and 65% O_2) modified $FADH_2$ oxidation activity caused a decrease in FAD fluorescence and induced an irreversible increase in ROS fluorescence. In these cases, the production of mitochondrial energy may be compromised, thus limiting human activities aimed to manage energy and also the environment envisioning a sustainable future.

The brain is particularly vulnerable to the effects of ROS and to changes in mitochondrial activity, because it is the biggest energy consumer. For this reason, it is important that society has this knowledge and practices breathing control in life's most challenging situations. It is important to promote social well-being, in order to reduce the amount of stress experienced at all levels, including in the family, school and work environments. This is essential to build a healthy and sustainable community able to safeguard energy and environment resources.

CRediT authorship contribution statement

S.M. Figueiredo: Investigation, Formal analysis, Validation, Writing - original draft. **F.O.C. Sousa:** Investigation, Writing - review & editing. **M.A.G. Lopes:** Investigation, Writing - review & editing. **R.M. Quinta-Ferreira:** Writing - review & editing, Project administration, funding acquisition. **M.E. Quinta-Ferreira:** Conceptualization, Supervision, Writing - review & editing, Project administration, funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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References

[1] Institute for sustainable communities. 1995, https://sustain.org/about/what-is-a-sustainable-community/. [Accessed 6 July 2020].
[2] Dan Buettner. The blue zones, second edition: 9 lessons to live more from the people who lived the longest. National Geogr Books 2012.
[3] Yun-Yi Ma, Chen Huei-Wen, Tzeng Chii-Ruey. Low oxygen tension increases mitochondrial membrane potential and enhances expression of antioxidant genes and implantation protein of mouse blastocyst cultured in vitro. J Ovarian Res 2017;10(47).
[4] Christiane Brahimi-Horn, Pouysségur Jacques. Oxygen, a source of life and stress. FEBS Lett 2007;581(19):3582–91.
[5] Patrick Maxwell, Wiesener Michael, Chang Gin-Wen, Clifford Steven, Vaux Emma, Cockman Matthew, et al. The tumour suppressor protein VHL targets hypoxia-inducible factors for oxygen-dependent proteolysis. Nature 1999;399:271–5.
[6] Gregg Semenza, Nejfelt Mary, Chi Suzie, Antonarakis Stylianos. Hypoxia-inducible nuclear factors bind to an enhancer element located 3' to the human erythropoietin gene. PNAS 1991;88(13):5680–4.
[7] Paul Schumacker. Hypoxia, anoxia, and O_2 sensing: the search continues. Am J Physiol Lung Cell Mol Physiol 2002;283(5):L918–21.
[8] Giancarlo Solaini, Baracca Alessandra, Lenaz Giorgio, Sgarbi Gianluca. Hypoxia and mitochondrial oxidative metabolism. Biochim Biophys Acta 2010;1797(6–7):1171–7.
[9] Aashish Jha, Zhou Dan, Brown Christopher, Kreitman Martin, Haddad Gabriel, White Kevin. Shared genetic signals of hypoxia adaptation in drosophila and in high-altitude human populations. Mol Biol Evol 2015;33(2):501–17.
[10] Fenglian Zhao, Yang Junling, Cui Ranjy. Effect of hypoxic injury in mood disorder. Neural Plast 2017;2017:698698.
[11] Dominik Fuhrmann, Brüne Bernhard. Mitochondrial composition and function under the control of hypoxia. Redox Biol 2017;12:208–15.
[12] Hye Hwang, Lynn Scott, Vengellur Ajith, Saini Yogesh, Grier Elizabeth, Ferguson-Miller Shelagh, et al. Hypoxia inducible factors modulate mitochondrial oxygen consumption and transcriptional regulation of nuclear-encoded electron transport chain genes. Biochemistry 2015;54(24):3739–48.
[13] Ioanna Papandreou, Cairns Rob, Fontana Lucrezia, Lim Ai, Denko Nicholas. HIF-1 mediates adaptation to hypoxia by actively downregulating mitochondrial oxygen consumption. Cell Metab 2006;3:187–97.
[14] Jonas Hahn, Kienhofer Deborah, Stoof Julia, Csepregi Janka, Reinwald Christiane, Maueroder Christian, et al. ROS is the boss. Free Radic Biol Med 2018;120:S64–5.
[15] Viktor Reshetnikov, Hahn Jonas, Maueröder Christian, Czegley Christine, Munoz Luis, Herrman Martin, et al. Chemical tools for targeted amplification of reactive oxygen species in neutrophils. Front Immunol 2018;9(1827).
[16] Debora Coimbra-Costa, Alva Norma, Duran Mónica, Carbonell Teresa, Rama Ramón. Oxidative stress and apoptosis after acute respiratory hypoxia and reoxygenation in rat brain. Redox Biol 2017;12:216–25.
[17] Yury Lages, Nascimento Juliana, Lemos Gabriela, Galina Antonio, Castilho Leda, Rehen Stevens. Low oxygen alters mitochondrial function and response to oxidative stress in human neural progenitor cells. PeerJ 2015;3. e1486.
[18] Rebecca Brown, Lockwood Alan, Sonawane Babasahed. Neurodegenerative diseases: an overview of environmental risk factors. Environ Health Perspect 2005;113(9):1250–6.
[19] Thomas Clanton. Hypoxia-induced reactive oxygen species formation in skeletal muscle. J Appl Physiol 2007;102(6):2379–88.
[20] Francesca Galeffi, Somjen George, Foster Kelley, Turner Dennis. Simultaneous monitoring of tissue Po2 and NADH fluorescence during synaptic stimulation and spreading depression reveals a transient dissociation between oxygen utilization and mitochondrial redox state in rat hippocampal slices. J Cereb Blood Flow Metab 2010;31(2):626–39.
[21] Luke Thomas, Ashcroft Margaret. Exploring the molecular interface between hypoxia-inducible factor signalling and mitochondria. Cell Mol Life Sci 2019;76:1759–77.
[22] Toru Sasaki, Awaji Takuji, Shimada Kazuyoshi, Sasaki Haruyo. Increased levels of reactive oxygen species in brain slices after transient hypoxia induced by a reduced oxygen supply. Neuropsychiatry 2018;8(2):684–90.