Effects of exposure of rat erythrocytes to a hypogeomagnetic field

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Abstract

BACKGROUND: Hypomagnetic fields can disrupts the normal functioning of living organisms by a mechanism thought to involve oxidative stress. In erythrocytes, oxidative stress can *inter alia* lead to changes to hemoglobin content and to hemolysis.

OBJECTIVE: To study the effects of hypomagnetism on the state of rat erythrocytes *in vitro*.

METHODS: Rat erythrocytes were exposed to an attenuated magnetic field (AMF) or Earth's magnetic field (EMF), in the presence of *tert*-butyl hydroperoxide (TBHP) as inducer of oxidative stress. Determinations: total hemoglobin (and its three forms - oxyhemoglobin, methemoglobin, and hemichrome) released from erythrocytes, spectral data (500-700nm); oxygen radical concentrations, electron paramagnetic resonance.

RESULTS: AMF and EMF exposed erythrocytes were compared. After 4h incubation at high TBHP concentrations ($> 700\ \mu M$), AMF exposed erythrocytes released significantly more ($p < 0.05$) hemoglobin (Hb), mostly as methemoglobin (metHb). Conversely, after 24 h incubation at low TBHP concentrations ($\leq 350\ \mu M$), EMF exposed erythrocytes released significantly more ($p < 0.001$) hemoglobin, with metHb as a significant proportion of the total Hb. Erythrocytes exposed to AMF generated more radicals than those exposed to the EMF.

CONCLUSION: Under particular conditions of oxidative stress, hypomagnetic fields can disrupt the functional state of erythrocytes and promote cell death; an additive effect is implicated.

Keywords: erythrocytes, haemoglobin, hypomagnetic field, reactive oxygen species, cell death

1. Introduction

All living organisms experience the effect of the Earth's magnetic field (EMF), which varies depending on the degree of latitude increasing to the poles and averaging 42 µT [1]. The magnitude of the induction of the magnetic field in near-Earth space is much lower than the magnitude of the magnetic field on the surface of the Earth and amounts to 0.0066 µT in interplanetary space, 0.3 µT at the lunar surface, ~ 0.7 µT at an altitude of 200 km from the surface of Mars [2-4].

Research progress into the biological effects of hypomagnetic fields has recently been reviewed [5]. Such research has potential relevance to the health and wellbeing of astronauts during aerospace travel and space station living. In a number of experiments it was shown that the use of a static magnetic field attenuated by screening (AMF) with induction of less than 20 µT violates the functional state of living organisms [6-9]. AMF can affect both the rate and duration of the cell cycle, and the effects of exposure to AMF on human lymphocytes are more significant in the G1 phase [10, 11]. Reducing the induction of the EMF to 300 nT leads to inhibition of proliferation and differentiation of skeletal muscle cells in newborn rats [9, 12].

It is assumed that the mechanism of the action of weak magnetic and electromagnetic fields in biological systems is associated with the generation of reactive oxygen species (ROS) [13, 14]. On the other hand, it has been found that intracellular production of hydrogen peroxide (H_2O_2) in cancer cells and in endothelial cells of arteries is suppressed under AMF [15, 16]. It is also assumed that the geomagnetic sensitivity of living organisms depends on the generation of the superoxide anion ($O^{2\bullet}$) [17, 18]. Hypomagnetic fields can modulate the interconversion of a singlet-triplet transition involving superoxide [18-20].

Generation of ROS by erythrocytes induces autoxidation, the essence of which is spontaneous oxidation of heme iron (Fe^{2+}). This process, if not controlled by the antioxidant defense system, leads to the formation of metHb (Fe^{3+}) and $O^{2\bullet}$ [21]. Superoxide can react

with hemoglobin (Hb) and undergo dismutation with the formation of H_2O_2, which can initiate lipid peroxidation and/or react with Hb [22]. If the antioxidant system of erythrocytes is disrupted, too much ROS are produced, oxidative stress leads to the destruction of red blood cells (hemolysis), Hb is released into the intravascular space and endothelial NO is consumed, resulting in spasm and hypertension [23]. In addition, oxidative stress causes increased erythrocyte rigidity as a result of eryptosis, which can lead to anemia and / or blood microcirculation disruption [23].

The purpose of the research reported on here was to study the effects of AMF on the state of rat erythrocytes *in vitro*.

2. Materials and methods

2.1 Reagents

HEPES and TEMPO (4-amino-2,2,6,6-tetramethylpiperidine-1-oxyl) were from Sigma (USA), all other reagents were from Vecton (Russia).

2.2 Preparation of red blood cells

Heparinized blood of Wistar rats was used. All manipulations with the animals were performed in accordance with the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978). Blood obtained after decapitation was centrifuged for 3 min at 3000 rpm and the plasma and lymphocyte-platelet layer were taken. The remaining erythrocyte mass was resuspended in Ca^{2+}-HEPES buffer (FC in mM: 140 NaCl, 3 $MgCl_2$, 10 Glucose, 10 HEPES, 2.5 $CaCl_2$ and 5 KCl) pH 7.4 and centrifuged twice for 2 minutes at 2000 rpm. One volume of Ca^{2+}-HEPES buffer was added to the washed red blood cells, resuspended and blood counts were performed on a hematologic analyzer (Medonic M16). The number of lymphocytes in the suspension was $<0.1 \times 10^9$/L, and platelets $<0.01 \times 10^9$/L. The resulting suspension was adjusted to an erythrocyte concentration of 0.5 10^{12}/L using Ca^{2+}-HEPES buffer and poured into test tubes, which were subsequently used in the work. *tert*-Butyl hydroperoxide (TBHP) was used to induce

oxidative stress. A working solution of TBHP was obtained by diluting a stock 7.7 M solution just before the experiment. The test tubes were incubated on an orbital shaker (BioSan, OS-20) at 100 rpm for 4 and 24 h. During incubation the tubes were kept in a horizontal position at a room temperature of 24 °C with exposure to EMF or AMF.

2.3 Determination of hemoglobin forms

After incubation some of the samples were transferred into separate tubes and centrifuged at 3000 rpm. A spectrum was obtained from each supernatant at wavelengths from 500 to 700 nm with 1 nm step in a cuvette of 0.8 cm width. Spectrum data were used to calculate the molar concentration of total hemoglobin and its three forms - oxyhemoglobin (oxyHb), methemoglobin (metHb) and hemichrome (HC) - using the appropriate molar extinction coefficients [24]. The Hb concentration was expressed in μmol per 0.2 ml of erythrocyte suspension at a concentration of $5 \cdot 10^{11}$/L.

2.4 Determination of oxygen radical concentration by a spin-trap method using electron paramagnetic resonance (EPR)

The production of ROS in cells after exposure to EMF and to AMF was investigated by adding 4-amino-2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPO) as a spin-trap at a concentration of 0.3 mM to the erythrocyte suspension before incubation. After 15 hours of incubation, the samples were frozen in liquid nitrogen. The spectra of the obtained lysate of the erythrocyte suspension were recorded on an EPR spectrometer (Bruker Elexsys E580), designed to study solids (including single crystals), liquids and aqueous solutions at 24.2 °C (297.2 K) with slow scanning (16 scans) in 30 s and 5 s wait before re-scanning.

Electron spin resonance (ESR) spectroscopy combined with a spin-trap method is based on the idea that certain compound with a molecular structure quite close to that of nitroxyl radical (spin-trap) is connecting with free short-living radical (e.g. hydroxyl radical, $^-$OH) forming a long-living nitroxide radical (spin inclusion complex). The EPR spectrum for this new stable complex is unique for this radical or for the family of such radicals. TEMPO is one of the

most extensively used spin-trap compounds since the 1960s [25]. It is difficult to quantify the amount of ROS in the intact biological system, because they are unstable and rapidly dismutate or react with adjacent molecules. ESR combined with a spin-trap such as TEMPO is a reliable, direct and fast method for measuring ROS. Nitroxide radical is a stable chemical and can be easily detected by ESR within biological tissues *in vitro* and *in vivo* [26, 27]. In the present research, ESR investigation is aimed at the $^-$OH detection.

2.5 Attenuation of the Earth's magnetic field in the shielding chambers

For physical modeling of an ultraweak static magnetic field, two shielding chambers were created in the form of cylinders (1st chamber: diameter = 10 cm, length = 30 cm, 2nd chamber: diameter = 25 cm, length = 60 cm), covered with tens of layers of shielding material manufactured from alloys of amorphous soft magnetic material AMAG172 (Kuznetsov et al., Russian Patent No: No. 2324989, 2008). All MF measurements were carried out using a Fluxmaster magnetometer (Stefan Mayer Instruments, Dinslaken, Germany) in the range of 1 nT - 200 µT with a resolution of 1 nT, and with magnetometer (HB0302.1A, St. Petersburg, Russia) in the range 0.1 -100 mT with resolution of 0.1 mT.

2.6 Statistics

Determination of hemoglobin forms was performed as 10 independent experiments. Blood was derived from one rat in each experiment (n = 10) and split into several samples which were exposed to either EMF or AMF. Three parallel measurements of each of the Hb forms, which were further averaged, were done per sample. Thus, each group (EMF or AMF) included 10 averaged measurements per Hb form. The statistical analysis was done using GraphPad Prism 7 (GraphPad Software, Inc., San Diego, CA). Data were analyzed using paired Student's t-test, and Benjamini-Krieger-Yekutieli false discovery rate correction procedure was applied [28].EPR spectra were further quantified using calculation of energy and average power over finite magnetic field strength interval. This computation was done

using spline (degree = 3) curve fitting to obtained spectra and subsequent integration (and normalization by magnetic field strength for power calculation).

3. Results

3.1 Hemoglobin in the supernatant of erythrocytes after exposure to EMF or AMF

Estimation of total Hb showed that samples incubated in the presence of relatively low concentrations of TBHP (up to 350 μM) for 4 h had released the same quantity of Hb while exposure to either the EMF or AMF (Fig. 1a). However, at high concentrations of TBHP (above 700 μM) samples incubated for 4 h released less Hb under EMF exposure than AMF exposed samples; Hb release was generally lower by nearly 12% with significant difference ($p<0.05$) for all concentrations (Fig. 1a). Conversely, after 24 h incubation, in samples that were incubated with low TBHP concentrations (up to 350 μM) under EMF the total Hb released was significantly higher (from 74 to 316%, $p<0.001$) compared to the supernatant of samples that were incubated with exposure to AMF (Fig. 1b). In the presence of high concentrations of TBHP (above 700 μM), Hb release in samples exposed to AMF was generally higher but the difference was insignificant.

Determination of three forms of Hb showed that after 4 h of incubation, the difference in Hb released for samples with high TBHP concentrations (above 500 μM) was mostly in the metHb form (approximately 52 to 72% of total Hb concentration). After 24 h of incubation, the difference in Hb released for samples with low TBHP concentrations (up to 350 μM) was mostly in the oxyHb form (46 to 68% of total Hb concentration), though metHb consisted a significant part of the total Hb. Interestingly, in the case of spontaneous oxidation of erythrocytes in the absence of TBHP, after 24 h of incubation the release of total Hb from cells incubated with exposure to AMF was 162% lower than for cells incubated with exposure to EMF ($p<0.01$) (Fig. 2a). The amounts of metHb and oxyHb in the supernatant of the samples after incubation with exposure to EMF were 457% ($p <0.001$) and 198% ($p <0.05$) higher, respectively, compared to those for AMF exposed samples (Fig. 2b and 2c).

3.2 The level of oxygen radicals in the supernatant of erythrocytes after exposure to EMF or AMF

Measurement of the relative amounts of oxygen radicals (mainly the hydroxyl radical and superoxide anion), by EPR using the TEMPO spin label, showed that in the absence of TBHP after 24 h incubation there was a significant decrease in the intensity of the spectrum for the lysate of the erythrocyte suspension under exposure to AMF, compared to that under exposure to EMF (Fig. 3a). This indicates that in erythrocytes exposed to AMF, a greater number of free radicals are formed than under normal EMF conditions. Conversely, however, in the presence of TBHP (100 and 250 μM), after 24 h incubation the increase in the intensity of the spectrum for the lysate from AMF exposed erythrocytes was more than that for the lysate from EMF exposed erythrocytes (Fig. 3b and 3c). In general, there was a dose-related increase of the intensity of label spectrum with increase in TBHP concentration (Fig.4). Paradoxically it may seem, but this clearly indicates that TBHP at relatively low doses suppressed oxygen radical formation in the erythrocyte suspension.

4. Discussion

We did not detect differences in the effect of relatively low TBHP concentrations after 4 hours under the AMF or EMF conditions. Similarly, there was no difference in the effect of high concentrations of TBHP after 24 hours. An elevated release of total Hb - mainly as metHb - after 4 hours of incubation under AMF conditions in comparison with EMF, in the presence of high concentrations of TBHP, indicates an increase in cell lysis, i.e. necrotic death of erythrocytes and, consequently, potentiation of the action of TBHP by the attenuated geomagnetic field. An elevated release of total Hb after 24 h of incubation under AMF conditions in comparison with EMF, in the presence of high concentrations of TBHP, is less prominent and insignificant, which is explained by the time-dependent fulfillment of the entire process. The most interesting phenomenon is elevation of Hb under conditions of EMF, both

in the absence and low concentrations of TBHP, when the oxyHb form slightly predominates, with metHb representing a significant part of the total Hb. In accordance with this, TBHP at relatively low doses suppressed oxygen radical formation in the erythrocyte suspension.

In our recent *in vivo* study on rats [29], there was no apparent effect of AMF on hematological parameters and, of particular note, there was no effect on the number and average volume of erythrocytes or the concentration of Hb within them. This does not imply the absence of the effect of AMF on the biochemistry of erythrocytes in the longer term, but may indicate either an ability through hematopoiesis to quickly replace damaged cells in the compensatory phase and/or the absence of manifestations of a weak effect of AMF on the hematological parameters within the limited time of the *in vivo* experiment. In addition, the absence of a visible AMF effect under the usual conditions of the animals existence does not mean that under the conditions of oxidative or other types of stress, the effect of AMF would not be manifested much earlier and stronger. Additivity, synergy or potentiation of different effects - acting stronger or weaker on various targets - could amplify the effect of AMF. Therefore, in recognition of the inherent limitations of an *in vivo* rat model, in the present work we developed an *in vitro* model to detect a possible effect of AMF on erythrocytes within a reasonable time frame. It should be pointed out in this context that no evidence for damage of erythrocytes was obtained in the previous *in vivo* experiments, due to application of generally available and low-specific methods of clinical biochemistry. After obtaining the novel *in vitro* data from the present research it is now pertinent to consider development of a novel methodology that could reveal slight shifts in the state of red blood cells. This is especially important in view of the need to monitor the health and wellbeing of astronauts during aerospace travel.

In the context of aerospace travel, arguments could be given against the use of rat erythrocytes instead of human erythrocytes in experimentation. Indeed, there are differences in membrane properties of erythrocytes in these two species, such as erythrocyte deformability, cell membrane stability, osmotic gradient ektacytometry (osmoscan) and aggregation tendency

[30-32]. Furthermore, human erythrocyte membranes have comparatively lower total phospholipid and cholesterol contents, contain lower proportions of acidic phospholipids and higher proportions of lysophosphoglyceride and sphingomyelin, and are relatively more fluid; some of these properties have been shown to correlate well with the lower V_{max} of the Na^+/K^+-ATPase of human RBC membranes [31]. Despite these differences, similar results have been obtained in studies of aging, antioxidant nutraceuticals etc. upon this ATPase and other enzymes and transporters of rat and human erythrocytes [33-36]. Since our previous *in vivo* experiments under AMF conditions was with rats [29], it was logical for the present *in vitro* research to continue the experimentation with rat RBC.

The method we applied here for assessment of ROS generation provides several advantages. Firstly, it allows direct quantitative assessment of ROS, which is not possible with other techniques. Secondly, this method does not require complicated subcellular fractions procedures, which may alter the status of ROS. ESR can also measure the net amount of ROS even in the presence of antioxidants. Among the shortcomings of the ESR method we have to mention non-resonance absorption of the microwave frequency by biological objects, which can provoke heating of the sample. In order to prevent this process we applied a special thermostating. In addition, ESR is a rather expensive and labour-intensive technique so future research should focus on its simplification, together with elaboration of alternative techniques such as calculation of complex non-invasive biomarkers based on single and simple physiological and/or biochemical indicators.

Exposure of the living cells to high concentrations of TBHP results in a significant increase in cytotoxicity, ROS generation and lipid peroxidation. TBHP generates *tert*-butoxyl (*t*-BuO•) and *tert*-butyl peroxyl (*t*-BuOO•) radicals. The oxidative processes induced by TBHP in erythrocytes lead to free radicals formation in the reaction between TBHP and cellular Hb, which are then immediately consumed in lipid peroxidation reactions [37]. In addition, high concentrations of TBHP (0.5 mM) cause a decrease in activity of catalase and SOD [38]. The antioxidant/prooxidant

effects are not only correlated with the molecular structure but also the distributive status in the reaction system [39]. From this point of view, one should take into consideration that the redox potential $\Delta\xi^{\circ\prime}$ of TBHP (t-BuO$_2^{\bullet}$/t-BuO$_2$H redox pair) is at the level of 1050 mV and LogP is about 1.23 [40, 41]. This means that at low concentrations of TBHP, this oxidant can accept electrons from oxygen radicals such as superoxide anion and hydrogen peroxide ($\Delta\xi^{\circ\prime}$ of this redox pair is about 360 mV [42]) in the hydrophobic phase, protecting the membrane of erythrocytes from being oxidized. Even in the case of slow development of prooxidant effects, they initiate development of eryptosis with rigid membranes not prone to be broken easily, as we observed after 24 h exposure to low doses of TBHP.

The mechanism of amplification of oxidative stress in AMF conditions can be explained in terms of cytotoxic power, which assumes calculation of the rate of development of cytotoxic effects per unit of time [43]. Superposition of the cytotoxic effect of high power (TBHP in high concentration) causing necrotic type of cell death, with the low cytotoxic effect of AMF exposure inducing apoptotic cell death, leads to an intensification and acceleration of the necrotic pathway. While the superposition of two relatively low-power influences, each inducing apoptotic pathways, primarily accelerates the development of apoptosis. To clarify the molecular mechanisms of interaction between TBHP and AMF, it is necessary to estimate the level of reduced glutathione, the activity of the main antioxidant protection systems of erythrocytes, the activity of NADPH oxidase and the system of transmembrane electron transport. These aspects are the foci of our further research.

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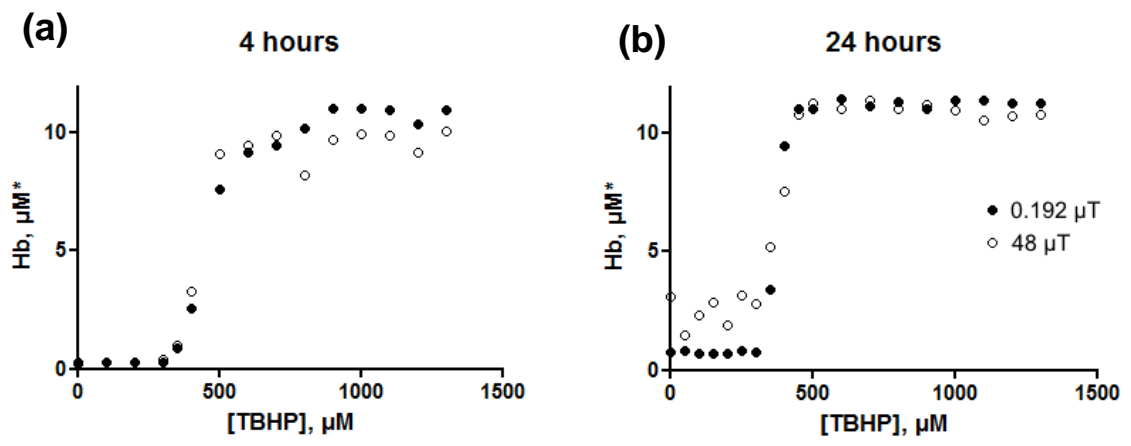
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Fig. 1. Release of total Hb (μM^* in 0.2ml suspension with 5×10^{11} RBC) into the supernatant of erythrocytes under exposure to EMF (48 μT) or AMF (0.192 μT), after 4 (a) and 24 h (b) h of incubation with various concentrations (0-1500 μM) of TBHP. Each point is the mean of at least three replicates; error bars are not shown due to the scale inconvenience.
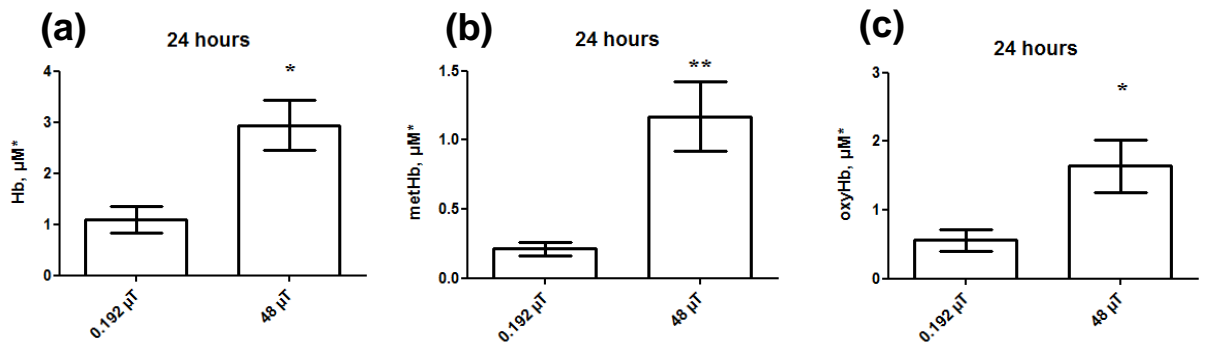
Fig. 2. Release of total (a), met- (b), and oxy- (c) hemoglobin (μM in 0.2ml suspension with 5×10^{11} RBC) into the supernatant of erythrocytes under exposure to EMF (48 μT) or AMF (0.192 μT) after 24 h of incubation in the absence of TBHP. * $p < 0.05$; ** $p < 0.01$.

Fig. 3. EPR spectra of the lysate of the erythrocyte suspension after 24 h incubation under EMF (48 μT) or AMF (0.192 μT) exposure, without TBHP (a) and with 100 (b) and 250 μM (c) TBHP.
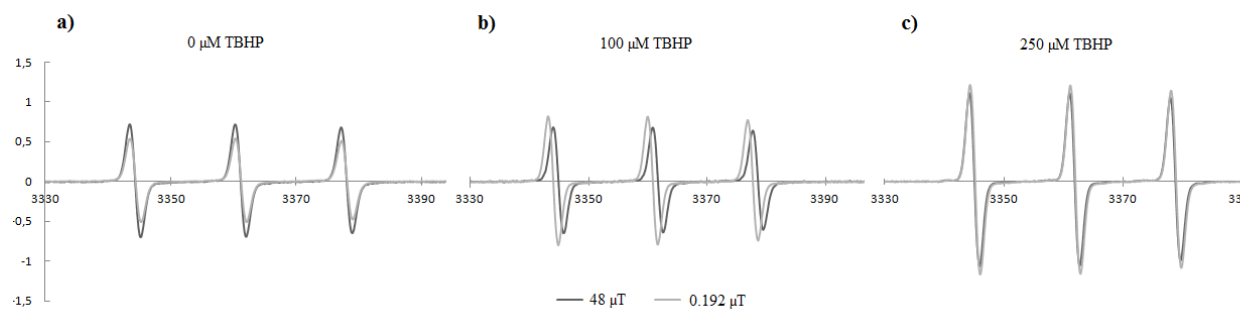
Fig. 4. Energy (a) and power (b) of EPR spectra of the erythrocyte suspension lysate after 24 h incubation under EMF (48 μT) or AMF (0.192 μT) exposure, without TBHP, and with 100, and 250 μM (c) TBHP.

Fig. 1

(a) 24 hours
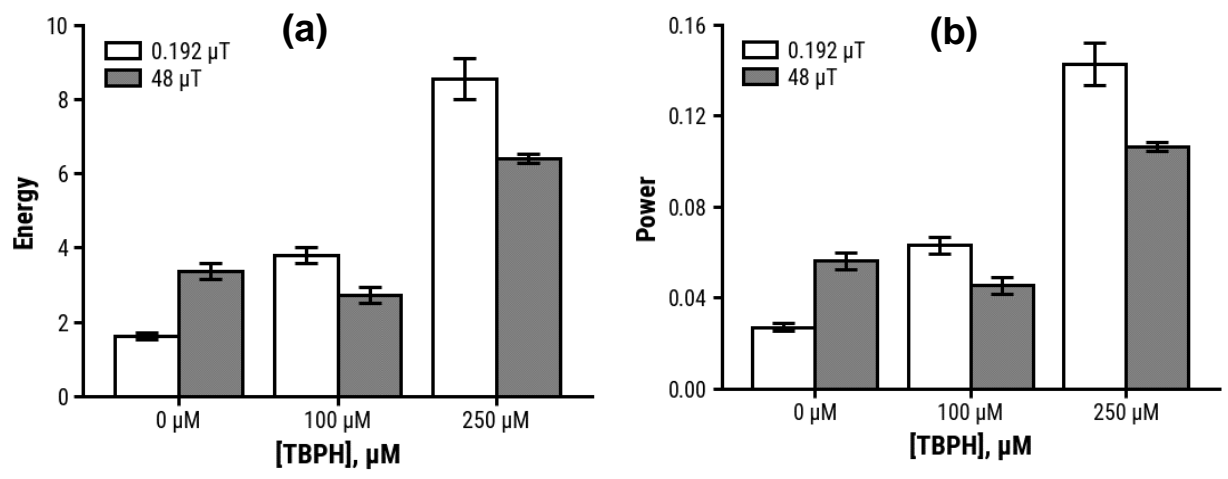
(b) 24 hours

(c) 24 hours

Fig. 2

Fig. 3

Fig. 4